The Endoplasmic Reticulum and Insulin Signalling in Adipocytes

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ABBREVIATIONS

ADP	adenosine diphosphate
APS	adapter protein containing a PH and SH2 domain
ATF6	activitting transcription factor 6
ATP	adenosine triphosphate
BMI	body mass index
CHO	chinese hamster ovary
CHOP	C/EBP-homologous protein
ER	endoplasmic reticulum
ERAD	ER-associated degradation
FCS	fetal calf serum
FOXO1	forkhead box O1
GAD	glutamic acid decarboxylase
GH	growth hormone
GK	glucokinase
GLUT	glucose transport protein
GLP	glucagon like peptides
GRB2	growth factor receptor-bound protein 2
Grp78/BiP	glucose regulated protein 78/immunoglobulin heavy-chain binding protein
GS3K	glycogen synthase 3-kinase
GSIS	glucose stimulated insulin secretion
GST	glutathione S-transferase
HDL	high density lipoprotein
HEK	human embryonic kidney
IGF	insulin-like growth factor
IGFR	insulin-like growth factor receptor
IKK	IκB kinase
IL	interleukin
IR	insulin receptor
IRE1	inositol-requiring protein 1
IRS	insulin receptor substrate
JMK	juxtamemberane
JNK	c-Jun N-terminal kinase

KRLB	kinase regulatory loop binding
MAPK	mitogen-activated protein kinase
mTOR	mammalian target of rapamycin
PBS	phosphate-buffered saline
PC	prohormone convertases
PDK1	PIP3-dependent protein kinase 1
PERK	protein kinase-like ER kinase
PH	pleckstrin homology
PIP2	phosphoatidyl-inositol-3, 4-bisphosphate
PIP3	phosphoatidyl-inositol-3, 4, 5-trisphosphate
PI3K	phosphatidyl inositol 3 kinase
PKB/Akt	protein kinase B
PKC	protein kinase C
PPAR-γ	perpxisome proliferator activated receptor-g
PTB	phospho tyrosine binding
PTPB1	protein-tyrosine phosphatise 1B
Ras	rat sarcoma
SDS	sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SH2	Src-homology domain 2
Shc	SH2-containing collagen-related protein
SHP2	SH2-domain-containing phosphotyrosine phosphatase 2
SREBPs	sterol regulatory element-binding proteins
TK	tyrosine kinase domain
TRAF2	tumour necrosis factor receptor-associated factor 2
TNFα	tumour necrosis factor alpha
TUNEL	terminal deoxynucleotidyl transferase-mediated dUTP nick-end-labelling
UPR	unfolded protein respons

SUMMARY

Upon binding of insulin, the insulin receptor (IR) is activated by phosphorylation on tyrosine residues. This engages insulin receptor substrate (IRS) proteins and transmits the signal downstream via e.g. mitogen-activated protein kinases (MAPK) and protein kinase B (PKB) to evoke context-specific responses like increased glucose uptake into muscle and fat and suppression of hepatic glucose production. Not only insulin sensing but also insulin production in pancreatic β-cells depends on IRS function. Disturbance of IRS signalling can result in diabetes.

Recent findings indicate that obesity-induced endoplasmic reticulum (ER) stress in liver and fat impairs IRS-dependent signalling. The cellular response to ER stress, called unfolded protein response (UPR), activates c-jun N-terminal kinase (JNK) leading to serine phosphorylation and inhibition of IRS1. Furthermore, ER stress can also induce apoptosis in β-cells which underlines the outstanding role of the ER for the proper regulation of blood glucose homeostasis.

Glucose-regulated protein 78 (Grp78/BiP) is a molecular chaperone involved in the folding of nascent polypeptide chains in the ER. It is also an important regulator of the UPR which controls transcription, translation and apoptosis upon ER stress.

Our lab had previously performed yeast 2-hybrid screening with IRS1/2 as bait and isolated several new IRS-binding proteins. Grp78/BiP was one of the candidates. A clone encoding amino acids 336-517 of Grp78/BiP has been isolated in our screen with IRS2 as bait. This fragment interacted with IRS1 and IRS2 in yeast and in extracts from mammalian cells. The regions on IRS1 that are recognized by Grp78/BiP span amino acids 270-517 and 974-1242.

The aim of this thesis was to define possible molecular mechanisms that link the UPR to insulin signalling and thereby contribute to the development of diabetes.

To this end, the role of ER stress in adipocytes and in the development of insulin resistance was studied as well as the role of Grp78/BiP in insulin signal transduction.

It was found that ER stress represses expression/activation of the IR and IRS. However, ER stress did not block insulin-stimulated glucose transport in adipocytes, probably due to reduction of the expression levels of AS160. Further more, lypolysis was reduced under ER stress. The secretion of adipokines from adipocytes was disturbed under ER stress. Pro-inflammatory IL-6 was elevated but leptin and adiponectin were reduced. Conditioned medium collected from

adipocytes under ER stress increased proliferation of INS-1E cells. Ectopic expression of IRS1 or IRS2 counteracted ER stress-induced effects.

Under homeostasis, Grp78/BiP reduced activation of IR and MAPK but induced PKB activation, in both CHO-IR and 3T3-L1 adipocytes. Under ER stress, Grp78/BiP restored PKB activation in 3T3-L1 cells.

The presented work shows that obesity-induced ER stress reduces insulin signalling and alters the secretion of adipokines in adipocytes. The binding between IRS and Grp78/BiP may contribute to these changes. However, the reduction of signalling and the changes in adipokine secretion do appear not to impair insulin action since control over lipolysis and glucose transport was unaffected. ER stress appears to affect systemic regulation of energy homeostasis but does not impair the metabolic function of the individual adipocyte. Importantly, some effects of ER stress might even support maintenance of glucose homeostasis.

ZUSAMMENFASSUNG

Nach Bindung von Insulin wird der Insulin-Rezeptor (IR) durch Phosphorylierung an Tyrosin-Stellen aktiviert. Dies ermöglicht Bindung von Insulin-Rezeptor-Substrat (IRS)-Proteinen. Die Signalübermittlung aktiviert untergeordnete Kinasen wie z.B. Mitogen-Activated Protein Kinase (MAPK) und Protein Kinase B (PKB) und führt schlussendlich zu Kontext-spezifischen Reaktionen wie beispielsweise vermehrter Glukoseaufnahme in Muskel und Fett sowie Unterdrückung der hepatischen Glukose produktion. IRS Proteine werden nicht nur für die Insulinwirkung gebraucht, sondern auch für die Insulinproduktion in den β-Zellen der Bauchspeicheldrüse. Eine Störung der IRS-Signalübertragung kann Diabetes verursachen.

Neue Erkenntnisse zeigen, dass durch Fettleibigkeit verursachte Störungen im Endoplasmatischen Retikulum (ER, ER Stress) die IRS-abhängige Signalübertragung in der Leber und im Fett beeinträchtigen. Die zelluläre Reaktion auf ER Stress, die UPR (unfolded protein response), aktiviert c-Jun N-terminal kinase (JNK), die durch Serin-Phosphorylierung IRS1 hemmt. Darüber hinaus kann ER Stress auch Apoptose in β-Zellen induzieren, was wiederum die wichtige Rolle des ER für die korrekte Regulierung der Blutzucker-Homöostase unterstreicht.

Glucose-regulated protein 78 (Grp78/BiP) ist ein Chaperon, welches an der Faltung von entstehenden Polypeptidketten im ER beteiligt ist. Es ist ebenfalls ein wichtiger Regulator der UPR, die Transkription, Translation und Apoptose unter ER Stress kontrolliert.

In unserer Gruppe wurden mehrere neue an IRS-bindende Proteine isoliert, darunter Grp78/BiP. Ein entsprechendes Fragment mit den Aminosäuren 336-517 zeigte Wechselwirkung mit IRS1 und IRS2 in Hefe und in Lysaten von Säugerzellen. Als Bindungsstellen für Grp78/BiP auf IRS1 wurden Aminosäuren 270-517 und 974-1242 identifiziert.

Das Ziel dieser Dissertation war es, mögliche molekulare Mechanismen zu definieren, die die UPR und die Insulin-Signalübertragung verbinden und somit zur Entwicklung von Diabetes beitragen könnten.

Zu diesem Zweck wurde untersucht welche Rolle ER Stress und die UPR in Adipozyten spielen und ob die Entwicklung der Insulin-Resistenz von Grp78/BiP and dessen Interaktion mit IRS abhängen könnte.

Es wurde festgestellt, dass ER Stress die Expression/Aktivierung des IR und die von IRS Proteinen unterdrückt. Allerdings blockierte ER Stress durch Insulin stimulierten Glukose-

Transport in Fettzellen nicht, wahrscheinlich wegen der Reduktion der Expression von AS160. Ferner war die Lypolyse unter ER Stress reduziert und die Sekretion von Adipokinen durch Fettzellen gestört. Entzündungsfördernde Faktoren wie beispielsweise IL-6 waren erhöht, hingegen waren Leptin und Adiponektin reduziert. Konditioniertes Medium, welches von Adipozyten nach ER Stress-Induktion gesammelt wurde, bewirkte eine starke Proliferation in INS-1 Zellen. Ektopische Expression von IRS1 oder IRS2 wirkte vielen ER Stress-induzierten Effekten entgegen.

Grp78/BiP reduzierte unter Homöostase die Insulinabhängige Aktivierung des IR und der MAPK, aber induzierte die Aktivierung von PKB in CHO-IR Zellen und 3T3-L1 Adipozyten. Unter ER Stress wirkte Grp78/BiP der Reprimierung von PKB in 3T3-L1 Zellen entgegen.

Die vorgestellte Arbeit zeigt, dass durch Fettleibigkeit induzierter ER Stress die Insulin-Signalübertragung hemmt und die Sekretion von Adipokinen in Adipozyten verändert. Die Bindung zwischen IRS und Grp78/BiP könnte zu diesen Veränderungen beitragen. Allerdings scheinen die Hemmung der Signalübertragung und die Veränderungen der Adipokin-Sekretion die Insulin-Wirkung in Fettzellen nicht zu beeinträchtigen, da die Kontrolle über die Lipolyse und der Glucose-Transport unbeeinflusst blieben. Zusammenfassend zeigen unsere Ergebnisse, dass ER Stress vielmehr die systemische Regulierung der Glukose-Homestase beeinflusst als die einzelnen Adipocyten. Wichtig ist, dass einige Auswirkungen des ER Stresses sogar die Aufrechterhaltung der Glukose-Homöostase unterstützen konnten.

1 INTRODUCTION

1.1 Blood glucose homeostasis

D-Glucose is metabolized to ATP via the glycolytic pathway and serves as an important source of energy. Under physiological conditions, the blood glucose concentration in circulation is tightly regulated between 5.0-7.2 mmol/L [1]. Many factors contribute to keep the blood value of glucose in this remarkably narrow range, of which hormonal regulation is most important. There are two types of metabolic hormones affecting blood glucose levels: catabolic hormones (such as glucagon and cortisol) increase blood glucose by stimulating the production from liver, and anabolic hormones (such as insulin, growth hormone (GH) and insulin-like growth factor (IGF)) decrease blood glucose by triggering uptake of glucose into skeletal muscle and adipose tissue and induce storage of energy in liver, muscle and fat cells [2]. Failure to maintain blood glucose homeostasis leads to different diseases, of which diabetes is the most prominent one.

1.2 Glucagon

Glucagon is a 29 amino acid peptide hormone that was discovered in the 1920's [3]. The secretion of glucagon is stimulated by hypoglycaemia and inhibited by hyperglycaemia or insulin [4,5]. Proglucagon is expressed in pancreas, brain and intestine. In ileum, proglucagon can be processed to oxyntomodulin, Glicentin and Glucagon like peptides (GLP-1 and GLP-2), while it is processed by prohormone convertase (PC) 2 to glucagon in pancreatic α-cells (see [6]). The liver is considered to be the main target regarding the metabolic action of glucagon and a rise in blood glucose concentration after glucagon release is due to stimulation of glycogen breakdown (glycogenolysis) and increased gluconeogenesis [7].

1.3 Insulin

1.3.1 Structure and synthesis

Insulin was discovered in 1922 by Banting & Best and is regarded as most central in controlling blood glucose concentration [8]. It is a polypeptide hormone that consists of two chains called A and B. Disulfide bonds hold these two chains together. The synthesis of insulin only occurs in

pancreatic β-cells. The insulin mRNA is translated to preproinsulin from which a signal peptide is cleaved during insertion into the endoplasmic reticulum (ER) generating proinsulin. Proinsulin consists of three domains: a carboxy-terminal A chain, an amino-terminal B chain and a peptide known as C peptide in between. In the ER, PC1/3 and PC2 convert the proinsulin into mature insulin by proteolytic cleavage. Both insulin and cleaved C peptide are packaged into vesicles in Golgi and accumulate in the cytoplasm.

1.3.2 Insulin secretion

The secretion of insulin from pancreatic β-cells is a complex process [9]. After a meal, blood glucose rises and stimulates pancreatic β-cells to secret insulin. A first rapid increase in circulating insulin is observed which is due to so called first phase insulin secretion. It depends on the action of incretins secreted by the gut postprandially and inhibits hepatic glucose production in liver. Incretins also further stimulate the later phase of insulin secretion, called the second phase. A widely accepted sequence of events leading to glucose-induced insulin secretion is as follows: Glucose enters β-cells via type 2 glucose transporters (GLUT 2). Glucokinase (GK) intracellularly phosphorylates glucose to glucose-6-phosphate that is subsequently oxidized to ATP. The resulting increase in the ATP/ADP ratio closes cell surface ATP-dependent K^+ channels leading to the depolarization of the cell membrane. As a result, voltage-dependent Ca^{2+} channels are opened and extracellular Ca^{2+} enters the cells. Increased amounts of intracellular calcium causes release of insulin stored in secretory vesicles [10].

1.3.3 Insulin function

As described above, insulin decreases the concentration of glucose in blood. However, in addition to its role in regulating glucose uptake, insulin also affects numerous other cellular functions such as fat and protein metabolism. It stimulates lipogenesis, amino acid transport and protein synthesis while diminishing lipolysis. Insulin also regulates gene transcription through transcription factors, e.g., peroxisome proliferator-activated receptor (PPAR) isoforms and sterol regulatory element-binding proteins (SREBPs) and forkhead box O1 transcription factors (FOXOs). It stimulates growth, DNA synthesis, and proliferation. Consequently, defects in

insulin signalling can affect many organs and tissues (see review [11]). The following section will focus on how insulin regulates glucose metabolism in target tissues.

1.3.3.1 Insulin stimulates storage of glucose in the liver

The main function of insulin in liver is to store energy in the form of glycogen by increasing glycogen synthesis and, at the same time, reducing glycogenolysis and gluconeogenesis. Insulin activates protein kinase B (PKB) via phosphatidylinositol 3-kinase (PI3K). PKB then phosphorylates and thereby inactivates glycogen synthase kinase 3 (GSK3), which is a repressor of glycogen synthesis [12]. Another target of PKB is FOXO1, which activates expression of glucose 6-phosphatase, the main enzyme hydrolyzing glucose-6-phosphate resulting in the creation of a phosphate and free glucose. PKB phosphorylates FOXO1 to prevent its translocation to the nucleus and in turn downregulate glucose 6-phosphatase and glucose output [13,14].

1.3.3.2 Insulin promotes uptake of glucose into muscle and adipose tissue

Since the lipid bilayer is impermeable for carbohydrates, the transport of glucose into the cells needs specialized glucose carrier proteins. There are at least 14 members in this family [15]. Expression of these GLUTs is regulated in a tissue- and stage-specific manner. In muscle and adipose tissue, insulin-stimulated glucose transport depends on GLUT4 while basal uptake depends on the GLUT1 isoform [16]. In the basal state, GLUT4 cycles between one or more intracellular compartments [17,18]. Upon insulin stimulation, the GLUT4 vesicles rapidly translocate to and fuse with the plasma membrane [19], which efficiently increases the uptake of glucose. The intracellular signal transduction pathways controlling GLUT4 translocation are described below.

1.3.4 Insulin signal transduction

A summary of insulin signal transduction is shown in Figure 1.

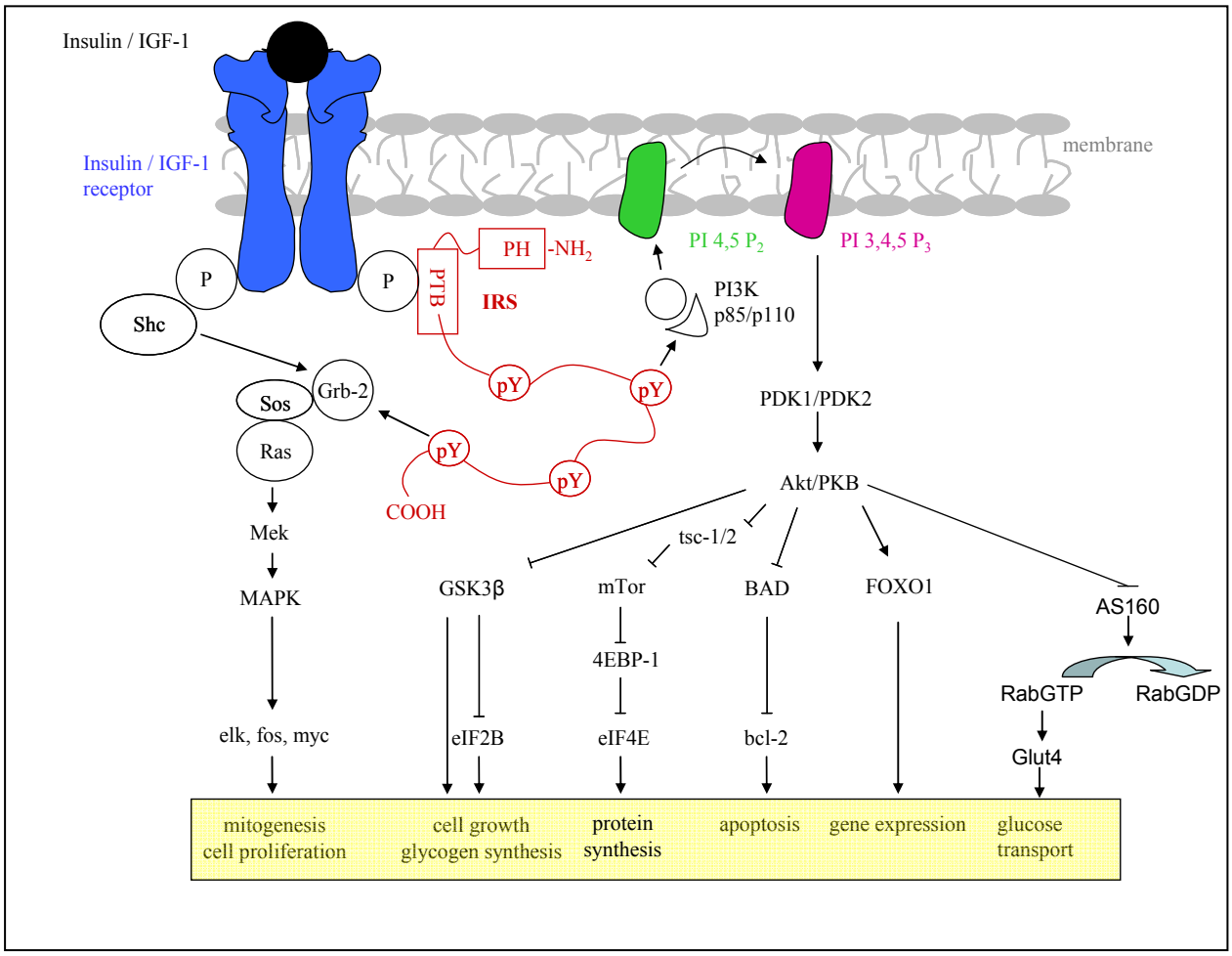

Figure 1. The insulin signalling network.

Kindly provided by Markus Niessen and Stephan Wüest.

1.3.4.1 Insulin receptor (IR)

The insulin receptor is a transmembrane protein composed of two α-and two β-subunits interconnected by disulfide bridges [20]. The α-subunits are entirely extracellular and bind to insulin, whereas the β-subunits span the plasma membrane (Figure 2). Four intracellular domains can be distinguished in the β-subunit. The juxtamembrane domain (JM) contains two tyrosine residues that are autophosphorylated in response to insulin binding [21]. The tyrosine kinase domain (TK) contains the enzymatically active site of the molecule, as well as the ATP-binding site and three important tyrosines (1158, 1162, and 1163) that must be phosphorylated for full kinase activity. Tyrosines in the C-terminus (CT) are not critical for receptor activation, but are thought to bind insulin receptor substrate 2 (IRS2) and to participate in the mitogenic effects of IR signalling. Binding of insulin to the α-subunit results in a conformational change and induces autophosphorylation of β-subunits (for a recent review on the IR see [22]. Following the phosphorylation/activation of the IR, intracellular signalling proteins are recruited and phosphorylated. Among these are members of the IRS and Shc family.

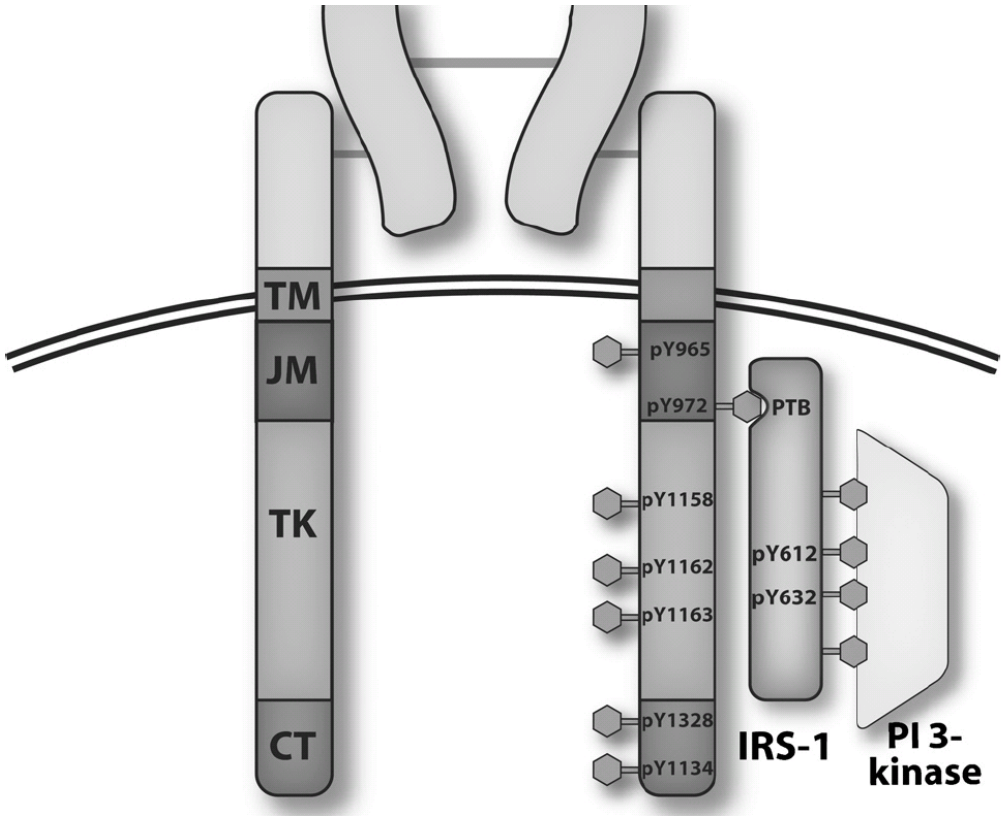

Figure 2. Schematic representation of the insulin receptor. Adapted from [22].

1.3.4.2 IRS proteins

IRS proteins act as adaptors and contain NH2-terminal pleckstrin homology (PH) and phosphotyrosine-binding (PTB) domains, followed by a variable COOH-terminal tail with multiple tyrosine and serine/threonine phosphorylation sites [23]. Upon insulin stimulation, the PH and PTB domains mediate binding between IRS and activated IR. Tyrosine phosphorylation motifs on IRS are specific substrates for insulin receptor kinase and other kinases. Tyrosin phosphorylation of IRS1/2 is essential for insulin signalling and provides binding sites for signalling proteins with SH2 domains such as phosphotidyl inositol 3 kinase (PI3K) and growth factor receptor-binding protein (Grb-2). On the other hand, serine/threonine phosphorylation of IRS usually negatively regulates signalling. Recruitment of downstream signalling components mediates the various insulin actions like cell survival, growth and differentiation. Serine phosphorylation of IRS1/2 represses insulin signalling. Not only insulin receptor but also other activated receptors as insulin-like growth factor receptor (IGFR) or interleukin-4 receptor (IL-4R) can recruit IRS proteins to transfer their respective signal to intracellular cascades. There are four major members in the IRS family (IRS1-4) all required for insulin signal transduction in different tissues [24].

IRS1 was identified first [25]. It contains 21 putative tyrosine phosphorylation sites and more than 30 putative Ser/Thr phosporylation sites. IRS1 localizes to the cytosol but binds to intracellular membranes upon insulin stimulation where it is found associated with PI3K [26,27]. In human adipocytes it has been found that IRS1 co-localizes with the IR in caveolae of the plasma membrane, both in basal conditions and after insulin stimulation [28,29]. IRS1 was found to be required for insulin sensitivity in muscle and adipose tissue [30], for insulin production from pancreatic islets [31] and for adipocyte differentiation [32,33].

IRS2 shares many characteristics with IRS1. Human IRS2 contains 22 potential tyrosine phosphorylation sites of which 13 are conserved in IRS1. IRS2 contains a unique region from amino acid 591-786 that interacts specifically with a kinase regulatory loop-binding (KRLB) domain of insulin receptor [34]. IRS2 is more concentrated in the cytosol. IRS2 is important for the metabolic function of liver and pancreatic β-cells [35-38]. It was shown that *irs2*$^{-/-}$ mice develop severe insulin resistance in liver in combination with failure to increase functional β-cell mass and even β-cell loss. Overexpression of IRS2 in pancreatic islets increases β-cell proliferation significantly, while IRS1 is less effective [35].

IRS3 was first detected in rat adipocytes as a tyrosine-phosphorylated protein of insulin receptor kinase [39]. IRS3 is located mainly at the plasma membrane, and activates PI3K in response to insulin. Humans lack a functional *irs3* gene, with two large deletions removing the PTB domain leading no long open reading frame in the remaining coding sequence. IRS3 is undetectable in human adipocytes [40].

IRS4 is expressed in various human tissues and cell lines [41]. However, IRS4 is a low abundance protein since it could not be detected in any tissue by standard immunological methods [42]. Overexpression of human IRS4 in rat adipocytes led to GLUT4 recruitment to plasma membrane independent of insulin [43]. Its overexpression reduced the phosphorylation of IRS1 and 2 [44].

1.3.4.3 PI3K and PKB/Akt

Metabolic insulin action typically depends on signalling through PI3K. PI3K contains two subunits: The p85 regulatory subunit acts as an adaptor and links the catalytic p110 subunit to the activated signalling complex [45,46]. When p85 binds to IRS1, the catalytic subunit p110 is activated and phosphorylates inositol biphosphate (PIP2) into triphosphate (PIP3) in the membrane. Two classes of serine/threonine kinases are known to act downstream of PI3K. One class is represented by Phosphoinositide-dependent kinase 1 (PDK1) and PKB/Akt, the second by the atypical protein kinase C isoforms ζ and λ (PKCζ/λ).

PKB/Akt is partly activated by phosphorylation of Thr 308 by PDK1 [47]. For full activation PKB/Akt also needs to be phosphorylated at Ser 403 [48]. Three isoforms of PKB/Akt exist in mammals (alpha, beta, and gamma) that control various biological functions such as cell survival, proliferation, cell growth, glycogen metabolism, and glucose uptake [49]. Stable expression of a membrane-bound form of PKBβ in 3T3-L1 adipocytes results in increased glucose transport and localization of GLUT4 to the plasma membrane [50]. Conversely, expression of mutated dominant negative PKB inhibits insulin-stimulated GLUT4 translocation [51,52]. In insulin resistant skeletal muscle, researchers found a relative decrease in insulin-stimulated association of IRS proteins with PI3K and activation of PKB/Akt [53].

1.4 Insulin resistance

Insulin resistance is defined as insufficient action of insulin in target tissues [54]. Early in 1939, Himsworth pointed out that the cause of some cases of diabetes is not deficiency of insulin but insensitivity of the tissues [55]. 10 years later, bioassays were developed to measure plasma insulin activity and they indicated that normal or even elevated insulin levels were present in some diabetic patients [56]. These discoveries then contributed to the classification of diabetes [57].

There are several causes of insulin resistance. An inherited component of insulin resistance in muscle has been defined in type 2 diabetic patients [58]. Other factors like physical inactivity, diet, hyperglycemia (gluco-toxicity), increased free fatty acids, and the aging process all contribute to the development of insulin resistance [59].

Under insulin resistance, signal transmission is blocked at one or several points [60,61]. For example, serine phosphorylation of IRS1 on specific residues (e.g. Ser307) inhibits its tyrosine phosphorylation and decreases the affinity of its binding to insulin receptor. Studies have shown that the cytokine tumour necrosis factor-α (TNF-α) [62], FFAs [63], c-Jun N-terminal kinase (JNK) [64], and nuclear factor-kappa B (NF-κB) [65] could induce serine phosphorylation of IRS thereby contributing to insulin resistance [66]. Besides impairment of IRS levels, other "critical nodes" might also be involved in the development of insulin resistance. One example is PIP3 that is down stream of PI3K could be obstructed by phosphatase and tensin homolohue (PTEN) therefore negatively affects insulin signalling. Moreover, Akt/PKB activation could be inhibited directly [67].

Insulin resistance in adipose tissue and muscle result in decreased insulin stimulated-glucose uptake and decreased suppression of gluconeogenesis as the read out of hepatic insulin resistance. These contribute to the rise of blood glucose level and diabetes development. Furthermore, under insulin resistance, adipocytes fail to reduce lypolysis by insulin and this will elevate the plasma FFAs. Chronical accumulation of FFAs in the circulation will impair other organ's (muscle, liver) insulin sensitivity as well as β-cell function [68].

1.5 Adipose tissue

Adipose tissue is well known as an organ that stores energy and secretes endocrine factors. It not only consists of adipocytes but also of other cell types such as macrophages, fibroblasts, blood cells and endothelial cells. In general, adipose tissue of mammals is found as loose subcutaneous connective-tissue layer between muscle and dermis as well as in the form of depots around heart, liver, kidney or lung [69]. Based on colour, adipose tissue is classified into two groups: white adipose tissue (WAT) and brown adipose tissue (BAT).

Cells from BAT contain mainly small lipid droplets as well as numerous mitochondria. Because BAT has reduced potential for oxidative phosphorylation, the energy produced in brown adipocytes is released as heat and not captured in the form of ATP [70]. This thermogenic process helps the body to adapt to cold environment. In contrast, WAT stores energy as triacylglycerol (TG) in large unilocular droplets, and releases it in the form of FFA [69].

1.5.1 Energy storage in white adipose tissue

FFAs are a major source of energy for many organisms. The energy produced from one gram fatty acids is 9 Kcal. Fat build-up is balanced by lipogenesis (fatty acids synthesis and subsequent TG synthesis) and lipolysis/fatty acid oxidation [71].

To compensate the fasting stage, cells store excess glucose from food intake as glycogen or fats droplets which contain water-insoluble TG. Compared to carbohydrate, less mass of fat can provide the same amount of energy, and quantitatively, fat is more important than glycogen in energy storage [72]. Although the cells of most tissues synthesise TG [73,74], in mammals, adipose tissue and liver are two of the most important pools of whole body TG. These two storage pools are functionally linked. Fatty acids exported from adipose tissue are the major substrates for hepatic very-low-density lipoprotein (VLDL) production and VLDL are returned to adipose tissue where FFAs are released by lipoprotein lipase [72].

FFAs are either derived from food or are synthesised from acetyl-coenzyme A (acetyl-CoA). After a meal, glucose levels are elevated and glycolysis occurs. Pyruvate from glycolysis is converted to acetyl-CoA and CO_2 within the mitochondria. The biosynthesis of fatty acids is catalyzed by fatty acid synthase (FASN) with acetyl-CoA as substrate [75]. The Triglycerides are then formed from a single molecule of glycerol, combined with three fatty acids (Figure 3).

To meet the metabolic need, fatty acids are released from TG to derive energy. This hydrolytic process is called lipolysis. In adipocytes, lipolysis can be dependent or independent of hormonal

regulation [76]. Hormone-sensitive lipase (HSL) is responsible for hormone-regulated lipolysis. It is activated by glucagon and hydrolyzes TG to diaglycerol (DG) and DG to monoacylglycerol (MG) [77].The mechanism of this reaction involves an increase in cyclic adenosine monophosphate (cAMP) which activates cAMP-dependent protein kinase (PKA) to phosphorylate HSL [78] and the lipid droplet surface protein perilipin. The latter prevents HSL from accessing the lipid droplet when unphosporylated. After phosphorylation by PKA, perilipin loses the ability to protect the lipid droplet from HSL and lipolysis occurs [79,80]. Triacylglycerol hydrolase (TGH) was initially purified from liver and it was shown to play a role in hepatic TG metabolism independent of hormonal regulation [81]. Cholesterol but not fatty acid feeding increases the TGH expression [82]. In adipocytes, TGH is considered as a regulator of basal lipolysis because inhibition of TGH reduces basal free fatty acid release from 3T3-L1 adipocytes [83].

Both lipogenesis and lipolysis are regulated by nutrition and hormones [71]. Insulin is one of the most important hormones to control the balance. It was found that insulin stimulates lipogenic genes in adipocytes [84]. Fat-specific insulin receptor knock-out produced less adipose tissue and protected from obesity [85]. On the other hand, insulin plays an opposite role in the regulation of HSL-induced lipolysis. Insulin induces phosphorylation and activation of the phosphodiesterase type 3B (PDE3B), leading to a decrease in cAMP levels and concomitant decrease of PKA activity [86,87].

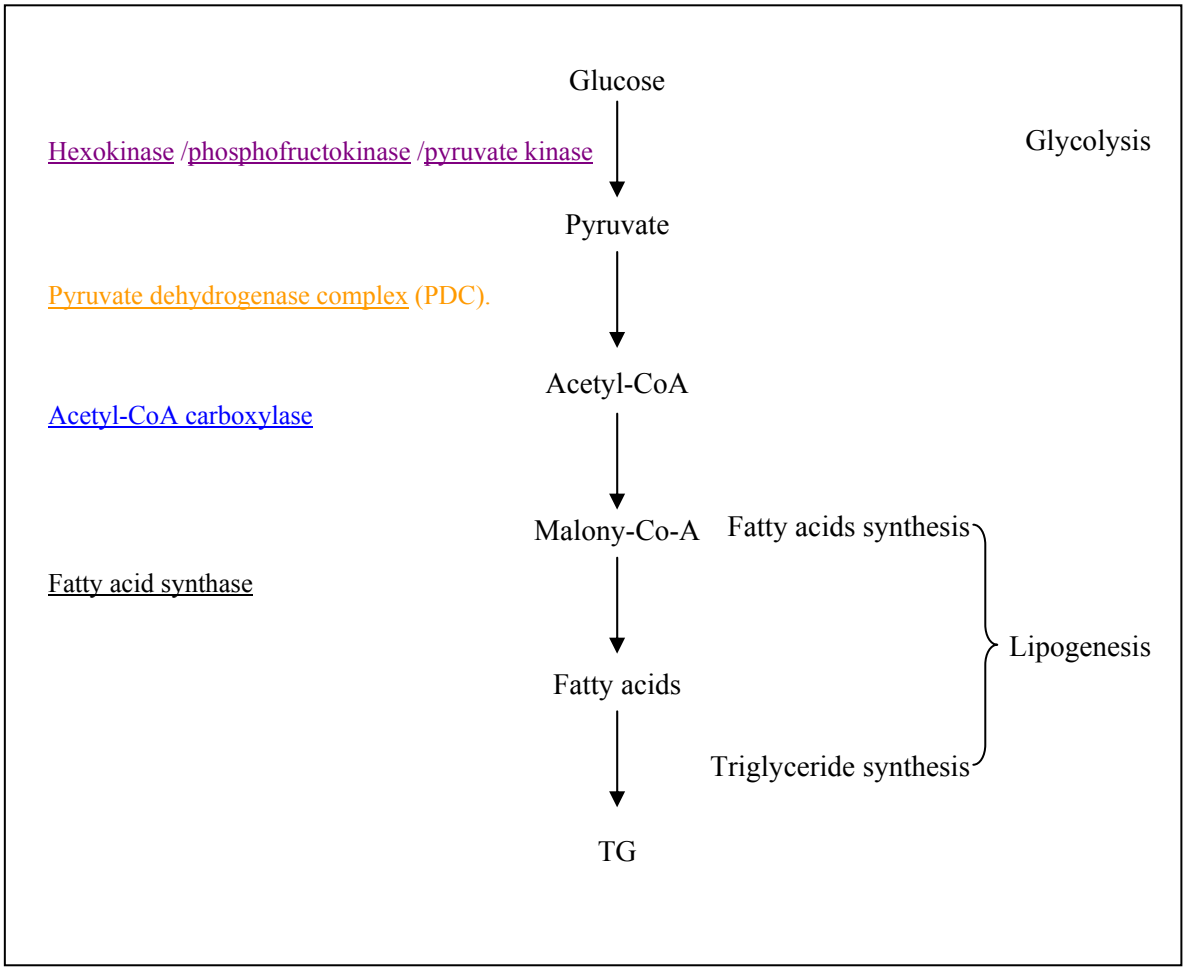

Figure 3. Glycolysis and lipogenesis. Adapted from [71].

1.5.2 Adipokines and insulin resistance

WAT is essential for glucose homeostasis because it secretes a great number of bioactive mediators, which are called adipokines [61,88]. These include leptin, adiponectin and pro-inflammatory cytokines like interleukin-6 (IL-6) and TNF-α. Leptin and adiponectin are grouped as anti-diabetogenic. Adiponectin can decrease TG synthesis and enhance insulin action in skeletal muscle and liver [89]. The role of leptin is complex. It affects energy homeostasis by reducing appetite and increasing thermogenesis via the hypothalamus [90]. It was also shown to be required for normal immune response [91] and to induce inflammation-related genes in insulinoma cells [92]. It was shown that animals lacking white adipose tissue suffer from severe insulin resistance in liver and muscle, with increased TG storage in these tissues. Transplantation of fat from normal mice to these animals could restore insulin sensitivity [93]. The homeostasis of the adipokines secretion is dependent on the stage of adiposity [69]. It has been shown that body mass index (BMI) correlates with the number of macrophages in adipose tissue [94] and during the development of obesity, adipose tissue-resident macrophages can switch the phenotype from anti-inflammatory (M2) to inflammatory (M1) [95,96]. Macrophages in WAT can also secrete factors that impair insulin signalling such as pro-inflammatory factors.

Obesity is the highest risk factor among the factors that are believed to contribute to the development of insulin resistance. More than 80% of subjects with type 2 diabetes are overweight [68]. When caloric intake exceeds energy expenditure, adipocytes increase their number (hyperplasia) and size (hypertrophy) to store the excessive energy [97]. Obesity is characterized by a state of chronic mild inflammation [98], with raised circulating levels of inflammatory markers. Accordingly, the expression and release of inflammation-related adipokines generally rises as adipose tissue expands. In 1993, TNF-α was shown to underlie insulin resistance in obese rats [99]. TNF-α activates IKKβ leading to serine phosphorylation of IRS1 and inhibition of insulin signal transduction. On the other hand, IKKβ can activate transcription factor NF-κB to stimulate IL-6 and TNF-α synthesis. Recently, obesity-induced ER stress in liver and adipocytes was considered as reason for insulin resistance in obese mice [100,101]. The molecular link was shown to be JNK, which can be activated under ER stress and phosphorylate IRS1 on Ser307.

The change of adipokine secretion in obesity not only influences insulin sensitivity of adipocytes but also affects other organs of the body (Figure 4). For example, elevation of IL-6 and TNF-α

plays a role in development of non-alcoholic fatty liver disease (NAFLD) that contributes to hepatic insulin resistance [102-104].

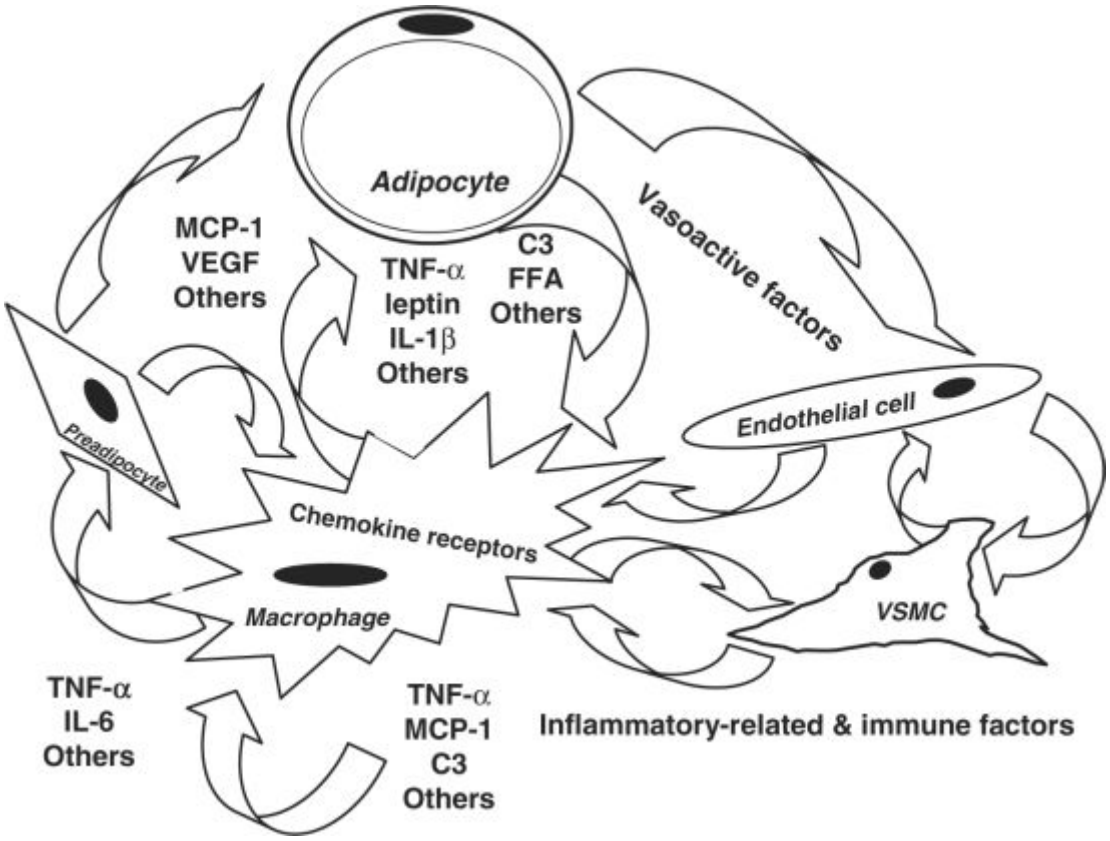

Figure 4. Autocrine-paracrine crosstalk underlying the complex adiposity-inflammation-immune relation. Adapted from [70].

1.5.3 Metabolic Syndrome and Obesity

Changes in the environment (such as arsenic or dioxin exposure), behaviour and lifestyle have resulted in increased occurrence of metabolic disorders [105] Central obesity (fat accumulated between the internal organs in the torso with resulting increase in waist size), insulin resistance, fasting hyperglycaemia, elevated triglycerides (TG), high blood pressure, and decreased HDL cholesterol are key features of the metabolic syndrome or syndrome X. It is a combination of disorders that increases the risk of developing cardiovascular disease and diabetes. The underlying causes of this syndrome are overweight/obesity, physical inactivity and genetic predisposition. Obesity is defined by the World Health Organization (WHO) as a body mass index (BMI) > 30 and results when energy intake exceeds energy expenditure. It is important to counter obesity by food restriction and physical activity [106] to reduce the risk of syndrome X. The adipose tissue in obesity becomes chronically inflamed and suffers from a variety of stresses such as endoplasmatic reticulum (ER) stress [101] and metabolic stress. As a consequence, stress signalling is induced that can itself cause insulin resistance thereby further aggravating the development of diabetes (reviewed by [107]).

1.6 Etiology and incidence of diabetes

Diabetes mellitus has become a public health problem during the past few decades. The WHO reports that more than 180 million people were suffering from diabetes in 2007 and that this number may double until 2030. In 2002, costs caused by diabetes and its complications were around 132 billion dollars [108] in the USA. Diabetes mellitus is a syndrome of impaired carbohydrate, fat and protein metabolism characterized by high blood glucose and dyslipidemia. It is caused by either absolute lack of insulin or by decreased sensitivity of insulin target tissues in combination with relative hypoinsulinaemia. To compensate for insulin resistance in liver, fat and muscle pancreatic β-cells can initially increase production of insulin. As the β-cells often fail to produce sufficient amount of insulin to maintain normal blood glucose levels in the face of persistent insulin resistance in target tissues, progression to overt diabetes ensues.

Chronic hyperglycaemia is accompanied by the development of diabetes-specific microvascular damage in the retina, kidney and peripheral nerves. As a result, diabetes is a leading cause of

blindness, end-stage renal disease and neuropathies. Major diabetic complications include atherosclerotic macrovascular disorders, which increase the risk of myocardial infarction, stroke and limb amputation.

1.7 Classification of diabetes

According to the American Diabetes Association there are four types of diabetes mellitus by etiological classification: type 1 diabetes, type 2 diabetes, other specific types of diabetes (genetic defects in insulin action, drug or chemical-induced diabetes, infection-induced diabetes and so on) and gestational diabetes [109].

1.7.1 Type 1 diabetes

5-10 % of cases are classified as type 1 diabetes. Type 1 diabetes is believed to result from autoimmune destruction of the pancreatic β-cells resulting in an absolute insulin deficiency [110]. The markers of the immune destruction include islet cell auto-antibodies, auto-antibodies to insulin and auto-antibodies to glutamic acid decarboxylase (GAD). When most islets have been destroyed and there is no longer sufficient insulin production to control the blood glucose, diabetes ensues. Specific β-cell destruction by apoptosis is the hallmark of type 1 diabetes [111,112]. There are several pathways linked to apoptosis in pancreatic β-cells. Studies have suggested that macrophages and activated T-cells are responsible for the β-cell destruction via synthesis of proinflammatory cytokines, such as IL-1β, TNF-α, IL-6, as well as nitric oxide [113]. IL-1β is a pro-inflammatory cytokine often produced by macrophages. It is produced within islets and exposure of human islets to IL-1β induces expression of Fas on β-cells, probably leading to apoptosis. Incubation of islets in the presence of IL-1Ra (antagonist of IL-1β) represses Fas-mediated apoptosis after IL-1β treatment [114].

1.7.2 Type 2 diabetes

Type 2 diabetes accounts for around 90% of all diabetes cases [115]. The major risk factor for type 2 diabetes is obesity [116]. However, a conclusive mechanistic link between obesity, insulin resistance and the development of the metabolic syndrome is still missing. The hallmarks of type 2 diabetes are both insulin resistance and insulin insufficiency. Proper regulation of normal blood glucose levels depends on the balance of sufficient insulin production/secretion relative to insulin sensitivity in liver, muscle and adipose tissues. Diabetes eventually develops when under prolonged persisting insulin resistance the pancreatic β-cells fail to adapt, not producing sufficient insulin for normal regulation. β-Cell failure involves decrease in β-cell mass and function [117]. There are genetic and environmental components in the events leading to β-cell failure. Chronic exposure of pancreatic islets to elevated nutrients induces β-cell dysfunction and death. It was concluded that β-cell dysfunction is a consequence of 'glucolipotoxicity' rather then exposure to a single nutrient alone [118].

1.7.3 Type 1.5 diabetes

There is evidence for an overlap between type 1 and type 2 diabetes (reviewed by [119]). Analysis of clinical data showed that an increased number of patients with type 1 diabetes are obese and insulin resistant, two states classically associated with type 2 diabetes. Moreover, some of the factors which cause β-cell failure in type 2 diabetes are also known to play a role in the development of type 1 diabetes. It was therefore proposed that the two classical types of diabetes may be two extremes of a continuum. On the one hand diabetes can be triggered by factors causing a strong autoimmunity reaction, on the other hand, the β-cells can be destroyed under obesity and insulin resistance-associated inflammatory and metabolic stress. The final stage of both type 1 and type 2 diabetes is loss of functional pancreatic β-cell mass.

1.8 The Endoplasmic reticulum (ER)

The Endoplasmic reticulum (ER) is a membrane-surrounded intracellular compartment. It stretches from the nuclear envelop in all eukaryotic cells [120] and it has several functions. First,

it constitutes the site of synthesis and folding/processing of transmembrane as well as secreted proteins [121]. Secondly, it helps to sort proteins to their functional destination [122]. In addition, most membrane lipid bilayers are assembled in the ER. Other functions such as formation of triglyceride droplets [123,124], storage of calcium ions (Ca^{2+}) [125,126] and nutrient sensing were also attributed to the ER [127].

The ER is the first station in the pathway of secretory proteins and is responsible for the quality control of protein folding. More than 30% of newly synthesized proteins are delivered to their final destinations by this pathway [128]. Proteins containing a special N-terminal ER signal sequence is imported into the ER. Once the signal sequence is synthesised by the ribosome, it is recognized by a signal-recognition particle (SRP) forming a SRP-ribosome complex. This complex then binds to SRP receptor located on the cytosolic surface of the ER and docks the ribosome-nascent chain complex at a translocator named Sec61 complex. After docking, soluble proteins are translocated in to the ER lumen, whereas transmembrane proteins are integrated into lipid bilayer [129].

Protein glycosylation also occurres in the ER [130]. As soon as the polypeptide chain enters the ER lumen, precursor oligosaccharide (composed of N-acetylglucosamine, mannose, glucose and containing a total of 14 sugars) is transferred to target asparagines catalysed by oligosaccharyl transferase. If not to be retained in the ER itself, after proper folding proteins are exported to the Golgi complex.

The ER is also a compartment where lipids are synthesised. ER membrane-resident transcription factor family sterol-regulatory element binding proteins (SREBP) regulate cholesterol homeostasis. When sterol levels are low, inactive SREBPs will be released from the membrane of the ER and translocate to Golgi. After cleavage they become activated transcription factors and upregulate cholesterol or lipid synthesis [131].

As described above, TGH is an enzyme that catalyzes lipolysis. Several studies show that TGH is an ER-resident protein [132,133]. TGH was indentified to be a glycosylated protein, which needs to be modified in the ER for its activity [134]. Mature TGH contains an ER retrieval signal at the C-terminus [135].

1.9 ER stress and the unfolded protein response

ER homeostasis depends on sufficient capacity of the folding machinery to process newly synthesized polypeptide chains. When the folding capacity of the ER falls short of the folding requirement, ER stress occurs and cells activate a specialized response, called the unfolded protein response (UPR reviewed by [136]). The UPR helps the cells to re-establish the balance by increasing the protein folding capacity through induction of ER-resident molecular chaperones. It also increases the size of the ER. Furthermore, by suppressing transcription and translation the UPR also reduces loading of the ER. The ER also has a clearing ability called ER-associated degradation (ERAD) [137]. ERAD is activated during ER stress and exports the unfolded proteins to the cytosol for proteasome-mediated degradation. If the UPR can not remedy the stress, cells finally undergo apoptosis.

Molecular chaperones and among them glucose regulated protein 78/ immunoglobulin heavy-chain binding protein (Grp78/BiP) play an important role in the protein folding process [138]. Grp78/BiP participates in the translocation of nascent polypeptide chains into the ER and also recognizes unfolded or misfolded proteins in the lumen of the ER. As an ER-resident protein Grp78/BiP contains a typical signal sequence at the NH2-terminus (to guide it into the ER) and a KDEL sequence at the C-terminus (to hold it in the ER). The ADP-bound form of Grp78/BiP has high affinity for other proteins while exchange of ADP for ATP releases the substrates and allows for further folding. Grp78/BiP cycles between monomeric and oligomeric states. Oligomeric Grp78/BiP forms a storage pool. Only monomeric Grp78/BiP associates with unfolded proteins. Accumulation of unfolded proteins in the ER lumen increases the monomeric Grp78/BiP pool.

The UPR signals across the ER membrane via three transmembrane proteins: type I transmembrane protein kinase endoribonuclease inositol-requiring protein-1 (IRE1), protein kinase-like ER kinase (PERK) and type II transmembrane protein activating transcription factor 6 (ATF6, reviewed by [139]). Grp78/BiP binds to IRE1 and PERK and inhibits their activation by preventing dimerization. By binding to ATF6, Grp78/BiP masks two Golgi localization sequences and thereby prevents translocation of ATF6 into the Golgi. Under ER stress, Grp78/BiP is released from these three sensors, resulting in oligomerization of IRE1 and PERK and translocation of ATF6 to Golgi complex and activation.

Mammals have two isoforms of IRE1 called IRE1α and IRE1β. When released from Grp78/BiP, IRE1 oligomerizes and becomes transphosphorylated [140], resulting in activation of

endoribonuclease activity in the COOH-terminal domain of IRE1 and alternative splicing of transcription factor XBP-1. Spliced XBP-1 mRNA encodes a transcriptional activitor (XBP-1s) while the product of unspliced XBP-1 mRNA is an inhibitor of UPR (XBP-1u). The IRE1 pathway regulates chaperone production, ERAD and hepatic lipogenesis [141]. In mammals, recruitment of tumour necrosis factor receptor–associated factor 2 (TRAF2) by phosphorylated IRE1 results in activation of c-Jun N-terminal kinase (JNK) and triggers insulin resistance [101].

Release of Grp78/BiP from and activation of PERK phosphorylates eIF2α and results in downregulation of overall translation. The activation of PERK under ER stress is reversible and as soon as the homeostasis within the ER is restored, PERK is dephosphorylated [142].

ATF6 translocates to the Golgi complex after Grp78/BiP is released from its ER luminal domain. It is cleaved by Golgi-resident proteases S1P and S2P releasing its cytosolic fragment. The fragment then translocates to the nucleus and induces the expression of genes encoding molecular chaperones like *Grp78/BiP* and *Grp94*.

1.10 ER stress and diabetes

As described above, type 2 diabetes often develops due to pancreatic β-cell dysfunction in the face of insulin resistance. ER stress can affect both, insulin action and production and is therefore believed to significantly contribute to the development of the disease.

1.10.1 ER stress and pancreatic β-cells

A highly developed ER is one of the characteristic features of β-cells, in line with their ability to respond with high insulin secretion to increasing blood glucose concentrations. Under insulin resistance the demand for insulin production is greatly increased which may result in β-cell overload and ER stress. Studies show that ER stress-induced apoptosis may be a cause of diabetes [100]. There are three apoptosis pathways triggered by ER stress. One depends on transcription factor C/EBP-homologous protein (CHOP) and can be mediated by IRE-1, PERK and ATF6. The second pathway is activated by JNK and the third one is caspase-12-dependent, an ER-associated caspase.

ER stress-induced apoptosis has not only been studied in β-cell lines (MIN6), but also in animal models. The Akita mouse is such an animal model for diabetes. Akita mice progressively develop hyperglycaemia and show loss of β-cells mass without obesity [143]. Gene analysis showed that Akita mice carry a single copy of a mutated allele of the insulin 2 gene and this causes missfolding of proinsulin and its accumulation in the ER leading to the progression to diabetes due to β-cells death [144,145]. Further more, it has been shown that free fatty acids and cytokines that are known to induce pancreatic β-cell death also cause ER stress [146,147]. Interestingly, mice lacking PERK have profound β-cell dysfunction and become severely diabetic, whereas mice with a mutation in the PERK phosphorylation site in eIF2a have fewer β-cells and develop diabetes [148,149]. These results suggested a role for ER stress in β-cell failure in type 2 diabetes. To compensate for the excess nutrient levels, the body needs more insulin synthesis and secretion. This could exceed the protein folding capacity of the ER in β-cells and activate UPR. PERK might be required to inhibit protein translation and might reduce ER stress under chronic, continued overload (Reviewed by [54]).

All this evidence suggested that ER stress contributes to the development of diabetes because it disturbs the regulation of functional pancreatic β-cells mass.

1.10.2 ER stress and insulin resistance

It was reported that hyperglycaemia in Akita mice was accompanied by increased expression of ER stress sensors [150] and that systemic expression of molecular chaperone oxygen-regulated protein (ORP150) could improve insulin sensitivity in these mice [151]. Finally, in 2004, a report by Ozcan highlighted the link between ER stress, obesity and type 2 diabetes. Authors showed that ER stress occurs in liver and fat of high fat diet fed ob/ob mice. Activation of the UPR in response to ER stress was shown to inhibit IRS1 via JNK-dependent serine phosphorylation leading to reduced insulin sensitivity and development of insulin resistance in liver [101].

Recent evidence [152] also shows that high glucose levels in type 2 diabetes can induce the intracellular production of glucosamine leading to defective N-linked glycosylation of Apolipoprotein B-100 (apoB-100, a major protein component of plasma lipoproteins is required for the synthesis and secretion of triglyceride-rich circulating lipoproteins such as VLDL) and induction of ER stress. Sustained ER stress induced by high glucose could turn on apoptotic response and down regulate insulin-stimulated glucose transport in L6 cells [153].

Since the first studies [154] linking ER stress to diabetes were published numerous more experiments have been performed and described [101,151], however, the role of ER stress in adipose tissue has not been described yet.

1.10.3 ER stress and obesity

The cause(s) for the occurrence of ER stress in obesity is (are) still unknown, but the possibilities were reviewed recently [155]. In summary, first, the elevation of FFA levels under obesity might contribute to the obesity induced ER stress. It has been shown that FFA can induce ER stress in hepatocytes [156], pancreatic β-cells [146,157] and macrophages [158]. Although there is no direct evidence yet for FFA inducing ER stress in adipocytes, it was shown that FFA can activate JNK [159]. Since JNK is also activated downstream of the UPR ER stress might be the link. Hosogai et al. [160] indicated that adipose tissue in obese mice shows signs of hypoxia and this might be another reason of obesity-induced ER stress because hypoxia is a known condition that

can induce ER stress in adipocytes [155,161]. ER stress may also be caused by the inflammatory state in obesity. As described above, in obesity, several inflammatory factors including TNF-α are elevated and TNF-α was shown to induce ER stress in mouse fibrosarcoma cells [162]. The fourth possible cause for ER stress is oxidative stress which is also found under obesity. Indeed, in obese mice and humans oxidative stress markers are elevated in adipose tissue [163]. It is the current hypothesis that many stress signals converges in a common pathway finally leading to the induction of ER stress [127].

1.11 Hypothesis and aims of the project

As mentioned above, IRS proteins can be recruited by different ligand-bound receptors (IR, IGFR and IL-4R) to mediate different context-dependent downstream effects. Our group had developed a working model to describe how receptor-depend signal specificity could be conferred by IRS proteins (Figure 5). We suggest that IRS may bind in a context-specific manner with other proteins to distinguish between different signals. Context-dependent phosphorylation patterns on IRS could constitute a "code" that is "read" via the formation of signal-specific protein complexes.

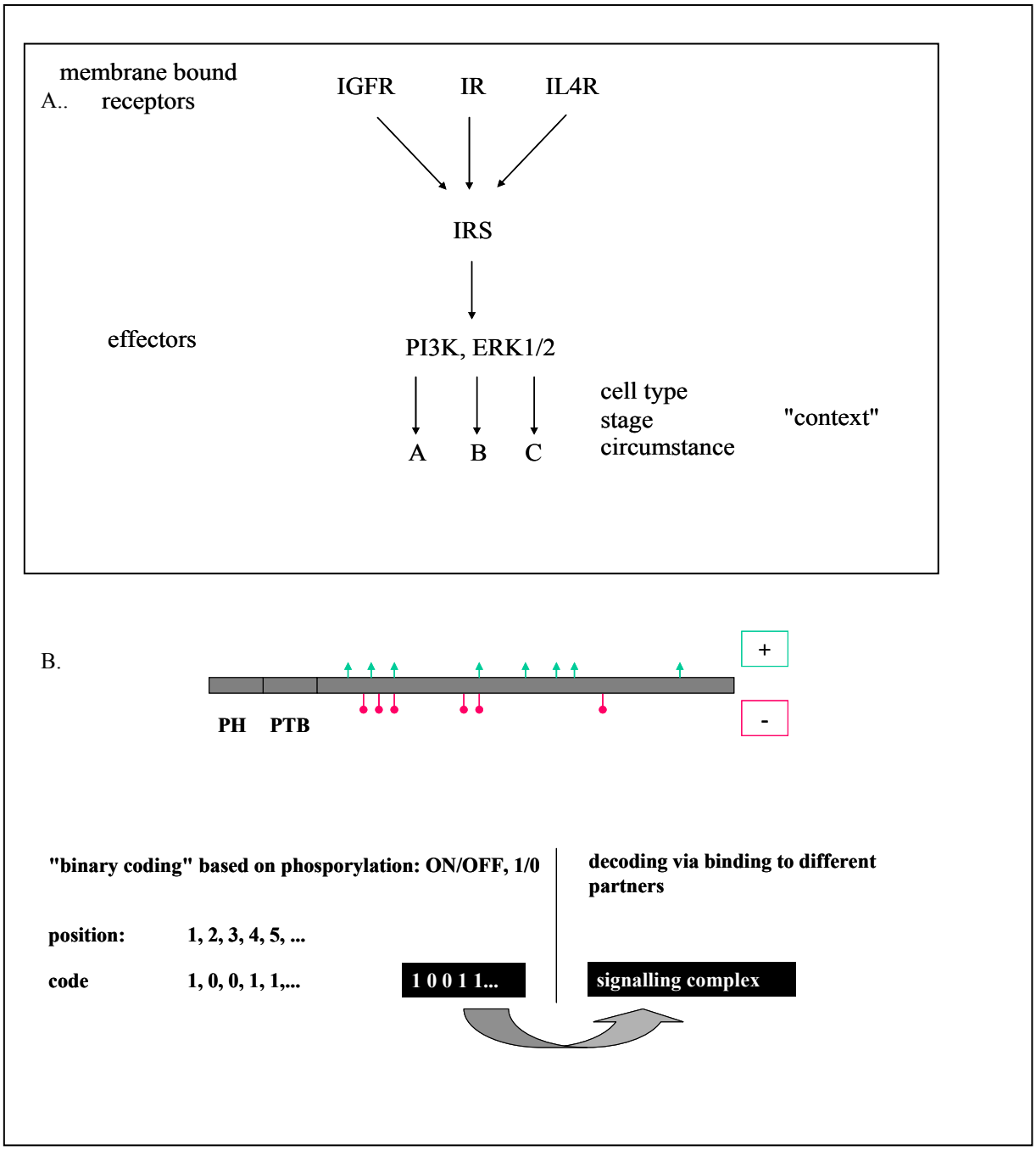

Figure 5. Alternative model describing the function of IRS proteins.

A. Different receptors send their signal via IRS to the same effectors. B. Coding-decoding model.

Based on our model yeast two-hybrid screening was performed to identify new binding partner of IRS. As a result, six proteins were identified (unpublished results) and their binding to IRS was confirmed by GST-pull down assays and immunoprecipitation experiments. One of the identified binding candidates was Grp78/BiP.

Our identification of the interaction between IRS and Grp78/BiP preceded the discovery by Ozcan et al. [101]. Once the link was established between ER stress, the regulation of blood glucose homeostasis and the development of type 2 diabetes the focus of our project was adapted and as a result this thesis consists of two parts.

Part 1 describes the effects of ER stress in adipocytes. Despite of the prominent role fat tissue plays in the development of the metabolic syndrome and although ER stress occurs in adipocytes during obesity, very little is known about how ER stress affects adipocytes. This first part is the main focus of our study.

Part 2 describes many of the experiments we had initially planned and deals with the interaction. This second part is based on the hypothesis that binding of Grp78/BiP to IRS1 and IRS2 could initiate the formation of a complex that links the UPR and IRS-dependent insulin signal transduction. In this sense, IRS proteins can be seen as downstream targets of the UPR.

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2 RESULTS

2.1 Part 1: ER Stress in adipocytes inhibits insulin signalling, represses basal lipolysis and alters the secretion of adipokines without inhibiting glucose transport

ER Stress in adipocytes inhibits insulin signalling, represses basal lipolysis and alters the secretion of adipokines without inhibiting glucose transport

Running title: ER stress in adipocytes

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ABSTRACT

The endoplasmic reticulum (ER) is the intra-cellular site, where secreted and membrane proteins are synthesized. ER stress and activation of the unfolded protein response (UPR) contribute to insulin resistance and the development of diabetes in obesity. It was shown previously in hepatocytes that the UPR activates c-jun N-terminal kinase (JNK) which phosphorylates insulin receptor substrate (IRS) proteins on serine residues thereby inhibiting insulin signal transduction. Here we describe how ER stress affects insulin signalling and the biological function of adipocytes. In addition to inhibition of IRS we found that ER stress downregulates the expression of the insulin receptor. Concomitantly, insulin-induced activation of Akt/PKB and of ERK1/2 was strongly inhibited. Ectopic expression of IRS1 or IRS2 counteracted the inhibitory effect of ER stress on insulin signalling. Similarly, pharmacological inhibition of JNK with SP600125 also improved insulin signalling under ER stress suggesting an inhibitory role of JNK downstream of the UPR. ER stress decreased the secretion of the adipokines adiponectin and leptin, but strongly increased secretion of IL-6. ER stress inhibited expression and insulin-induced phosphorylation of AS160, did not inhibit glucose transport but reduced lypolysis. Finally, supernatants collected from 3T3-L1 adipocytes undergoing ER stress improved proliferation when used to condition the culture medium of INS-1E β-cells. It appears that ER stress in adipocytes leads to changes resembling early pre-diabetic stages which at least in part support the regulation of systemic energy homeostasis.

Key words: ER stress, insulin signalling, insulin resistance, adipocytes, adipokines

Introduction

The endoplasmic reticulum (ER) is a complex intra-cellular membranous network that forms the site of synthesis, processing and sorting for ER-resident, membrane-bound and secreted proteins. Deterioration of ER homeostasis can impair the regulation of blood glucose levels since insulin secretion from pancreatic β-cells [1] and insulin action both depend on the proper functioning of the ER. Importantly, it was shown that obesity which is associated with the pathogenesis of type 2 diabetes impairs proper function of the ER (ER stress) in fat and liver cells causing insulin resistance in hepatocytes [2]. ER stress occurs when unfolded proteins accumulating in the lumen of the ER activate a cellular emergency program to re-establish homeostasis called unfolded protein response (UPR). The presence of unfolded proteins in the ER is sensed and activation of the UPR is regulated via the ER chaperone Grp78/BiP. The UPR triggers complex intracellular events including upregulation of chaperones (e.g. Grp78/BiP) to increase folding capacity, selective downregulation of translation, protein degradation and regulation of apoptosis. Importantly, ER stress activates c-jun N-terminal kinase (JNK, [3]) which is a known inhibitor of insulin receptor substrate (IRS) proteins [4,5], and it was found that obesity-dependent ER stress in liver cells inhibits insulin action by downregulation of IRS1 [2]. IRS proteins bind to activated insulin receptors and relay the insulin signal to downstream pathways such as the phosphatidyl inositol-3 kinase (PI3K)/protein kinase B (Akt/PKB) and mitogen-activated protein kinase (MAPK p44/42 or ERK1/2) modules [6]. Activation of Akt/PKB increases glucose transport in muscle and fat tissue via phosphorylation/inhibition of AS160, a Rab GTPase activating protein that represses Glut4 translocation when in its non-phosphorylated form [7-9].

Factors synthesized and secreted by adipose cells are collectively called adipokines. They control systemic glucose and lipid metabolism, food intake, vascular homeostasis and immune response [10-12]. Among adipocyte secretory proteins and peptides are proinflammatory cytokines like IL-6 [13]. Chronically elevated levels of IL-6 are associated with the development of type 2 diabetes [14-16] and it has been found to inhibit insulin secretion from pancreatic islets [17] as well as insulin action in the liver [18-20]. Two other important products secreted into circulation by adipocytes are adiponectin and leptin [21,22] that either negatively or positively correlate with body fat mass, respectively.

The importance of ER homeostasis for systemic metabolic regulation is well established [23-27] but only few data are available on the metabolic consequences of ER stress and the UPR in adipocytes [28]. However, the molecular consequences of ER stress in adipocytes relating to insulin signal transduction and insulin action have not been described yet. Therefore, the aim of our study was to elucidate the effects of ER stress on insulin signalling and function in 3T3-L1 and primary mouse adipocytes. We studied expression and activation of components of the insulin signalling pathway, lipolysis, insulin-induced 2-Deoxy-D-[1-^{14}C] glucose uptake, glucose incorporation, and the secretion of selected adipokines after chemical induction of ER stress or under homeostasis. We also used conditioned medium to assess if and how changes in secretion might affect proliferation of INS-1E β-cells. Our findings indicate that ER stress in adipocytes can strongly inhibit insulin signalling and disturb the secretory function. However, not all consequences of ER stress appear to negatively affect metabolic regulation as demonstrated by unaffected glucose transport, repression of lipolysis and the promotion of β-cell proliferation.

Experimental Procedures

Cell Culture

Human Embryonic Kidney (HEK) 293 cells (gift from the research laboratory for Calcium Metabolism, Klinik Balgrist, Zurich, Switzerland) and 3T3-L1 fibroblasts (purchased from ATCC, Manassas, USA) were cultured in DMEM (4.5 g/L D-glucose) medium (Invitrogen) containing 10 % (v/v) FCS, 100 U/ml penicillin, 100 μg/ml streptomycin. CHO-IR cells (obtained from J. E. Pessin, University of Lowa, USA) were maintained in F12 medium (Invitrogen) containing 10 % FCS, 100 U/ml penicillin, 100 μg/ml streptomycin. INS-1E cells [29] were obtained from Pierre Maechler and Claes Wollheim (University of Geneva, Switzerland) and cultured in RPMI 1640 medium containing 11 mmol/l D-glucose, 10 % FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamax, 1 mM sodium pyruvate, 10 mM HEPES and 50 μM β-mercaptoethanol (Invitrogen, Carlsbad, California, USA). Cells were incubated at 37 °C, under 5 % CO_2 in a humidified atmosphere.

Differentiation of 3T3-L1 fibroblasts into adipocytes and ectopic expression

Fibroblasts were seeded on gelatin-coated plates and cultured in growth medium. Upon reaching confluence, differentiation was induced by incubating cells in differentiation medium (growth medium containing 0.5 mM isomethylbutylxanthine, 1 μM dexamethasone and 1.7 μM insulin) for 3 days. Differentiation medium was replaced with growth medium, supplemented with 240 nM insulin for an additional 3 days. Cells were kept in DMEM (4.5 g/L D-glucose) containing 2 % FCS for 5-7 days followed by DMEM (1.0 g/L D-glucose), 2 % FCS for 5 days prior to the start of an experiment.

Ectopic expression - Adenoviral constructs for ectopic expression of IRS1, IRS2 or GFP (as a control) have been previously described [30]. In brief, viral particles were purified from HEK293 cells 3-5 days post infection. Detached cells were harvested, lysed and infectious particles were purified by a centrifugation-based method. Ectopic expression was monitored with an antibody (4E10) against the myc tag.

Cell lysis and protein determination

Cells were washed three times in ice cold PBS and the last rinse was aspirated completely. Afterwards, lysis buffer (50 mM HEPES pH 7.5, 140 mM NaCl, 1 mM PMSF, 0.5 % Triton X-100, 10 mM NaF, 1 mM $Na_2H_2P_2O_7$, 1 mM Na_2O_4V, 3 μg/ml aprotinin, 3 μg/ml leupeptin) was added and cells were detached with a scraper. Lysates were transferred to Eppendorf tubes and incubated at 4 °C for 1 hour on a roller followed by centrifugation at 15,000 g for 20 min at 4 °C. Supernatants were transferred into fresh tubes and protein concentration was determined using the bicinchoninic acid (BCA) protein assay kit (Pierce).

Immunoblotting

Equal amounts of protein were separated on SDS-PAGE gels (NuPAGE, Invitrogen) and transferred onto PVDF membranes. 2 % non-fat milk in TBST (10 mM Tris-HCL, pH 7.4, 150 mM NaCl, 0.05 % (v/v) Tween 20) was used to block nonspecific binding of antibodies to membranes. Incubation with indicated primary and secondary antibodies was either at room temperature for 1 hour or overnight at 4 °C. Immuno-reactive proteins were visualized by the Lumi-Light Western Blotting Substrate (Roche) using the LAS-3000 imaging system (Fuji). Where appropriate signal intensities of the respective immunoblots were quantified using the AIDA software package (Raytest). Equal loading and transfer was confirmed with an antibody against actin (MAB1501, Chemicon International).

Induction of ER stress and assessment of insulin signalling

To induce ER stress cells were washed and supplemented with fresh medium containing appropriate concentrations of either tunicamycin (Applichem) or thapsigargin (Calbiochem), respectively. Incubation time and concentrations used are specified for each experiment within the text or in figure legends. Tunicamycin was dissolved at 20 mg/ml in DMSO and thapsigargin at 5 mg/ml in ethanol. Induction of ER stress was monitored by immunoblotting using a polyclonal antibody raised against Grp78/BiP (H129 from Santa Cruz). Insulin stimulation: Cells were starved (in culture medium containing 0.5 % BSA but no FCS) for two hours, stimulated with insulin (Sigma), washed on ice, lysed and protein concentration was determined. Where required, tunicamycin or thapsigargin were included in the starvation medium and during stimulation. Expression and activation of signalling components was determined by immunoblotting. Antibodies used to detect expression and activation of signalling components: IR, C-19 (Santa Cruz) and anti-phosphotyrosine (mouse monoclonal IgG2bk, Upstate Biotechnology). IRS1, C-20 (Santa Cruz). IRS2, 06-605 (Upstate Biotechnology). Akt/PKB, monoclonal anti-Akt/PKB (BD transduction Laboratories) and anti-phospho-Akt (Ser 473, Cell Signalling). ERK1/2 (p44/42), anti-p44/42 Map Kinase (#9102) and anti-phospho-p44/p42 Map Kinase (Thr202/Tyr204, #9101S) both from Cell Signalling. Anti-phospho-c-Jun (Ser73, Cell Signalling). AS160, anti-AS160 (#07-741) and anti-phospho-AS160 (#07-802) both from Upstate. Inhibitors: JNK, SP600125 (ALX-270-339, Alexis) dissolved in DMSO. PI3K, wortmannin (Sigma) dissolved in DMSO.

2-Deoxy-D-[1-^{14}C] glucose uptake and D-[U-^{14}C] glucose incorporation

3T3-L1 fibroblasts were seeded on 24 well plates, differentiated as described and, if required, transfected with adenoviral constructs. Cells were starved in 3T3-L1 starvation medium (DMEM 1 g/L glucose and 0.5 % BSA) for 2 hours. Uptake was initiated by the addition of 100 nCi/well of 2-Deoxy-D-[1-14C] glucose (GE Healthcare) along with the indicated amounts of insulin. Following a 30 minute incubation period, reactions were stopped by replacing the medium with ice-cold PBS. The cells were immediately washed three times in ice cold PBS and lysed as described. Lysates were analyzed for the expression of transgenes by SDS-PAGE and Western

blotting. For the determination of glucose uptake, the radioactivity in lysates was measured by liquid scintillation counting (Kontron Betamatic V).

Isolations of white adipocytes from 8-10 weeks old C57BL6 mice and glucose incorporation experiments were essentially performed as described previously [31,32]. Pooled adipocytes from 6-9 mice were incubated in Kreb's Ringer buffer containing 1 % BSA in the presence of DMSO (control) or tunicamycin (to induce ER stress) for 8 hours. Wortmannin was added 30 min prior to insulin and D-[U-^{14}C]glucose. The radioactivity in lysates was measured by liquid scintillation counting (Kontron Betamatic V).

Lipolysis

3T3-L1 adipocytes were cultured in the presence of Tu (2 μg/ml) or Thap (300 nM) for 18 hours, primary white adipocytes in the presence of Tu (10 mg/ml) for 4 hours. Insulin (100 nM) or isoproterenol (10 μM) were added where appropriate.

Lipolysis was assessed by measuring the concentration of glycerol in culture media of 3T3-L1 adipocytes or primary mouse white adipocytes for 1 hour in the presence of insulin or isoproterenol, essentially as described by [33].

Conditioned medium and proliferation of INS-1E cells

Differentiated 3T3-L1 adipocytes were incubated in the presence or absence of Tu or Thap for 18 hours. After three washes with PBS cells were incubated in Kreb's Ringer buffer without substance but containing 1 % BSA for two hours. Conditioned medium was prepared by diluting the Kreb's Ringer buffer into RPMI (2:3). Final concentrations of FCS and D-glucose were adjusted to 0.1 % and 6 mM, respectively. INS-1E cells were starved in serum-free RPMI for 4 hours followed by incubation in conditioned medium for 18 hours. [^{3}H]-thymidine was added and incorporation was allowed to proceed for 4 hours. Finally, cells were washed in ice cold PBS and lysed in trichloric acid (TCA). Incorporated radioactivity was measured by liquid scintillation counting (Kontron Betamatic V).

Adipokines

3T3-L1 adipocytes were cultured and treated as described in the legend to Fig. 6. After each experiment, cells were lysed and the induction of ER stress was confirmed by Western blotting. Adiponectin concentrations in supernatants were assessed using the Mouse Adiponectin ELISA Kit from AdipoGen (Korea). Concentrations of Leptin and IL-6 were determined using multiplex panels from Linco Research (Labodia, Switzerland).

Statistical Analysis

Data are expressed as mean ± SEM unless indicated otherwise and were analyzed by unpaired two-sided Student's t-test or by ANOVA, where appropriate. $p < 0.05$ was considered significant.

Results

Induction of ER stress in 3T3-L1 adipocytes

The optimal conditions for the induction of ER stress in 3T3-L1 adipocytes were determined. Cells were cultured in the presence or absence of tunicamycin (Tu, 2-10 μg/ml, inhibits glycosylation) or thapsigargin (Thap, 300-1000 nM, disturbs Ca^{2+} homeostasis) for different time periods and the expression of the commonly used ER stress marker Grp78/BiP was assessed by Western blotting to monitor the induction of ER stress. Both substances induced ER stress in a concentration- and time-dependent manner with thapsigargin being more potent and faster acting as compared to tunicamycin. A representative time course experiment is shown in Fig. 6A. Cells incubated in the presence of 1 μM thapsigargin or 10 μg/ml tunicamycin displayed increased expression of Grp78/BiP already after 4 or 6 hours, respectively. Longer incubations at reduced concentrations (2 μg/ml tunicamycin or 300-600 nM thapsigargin) were also effective (not shown here but see Fig. 7C, 8 and 9B/C)).

Fig. 6B shows that c-jun which is a target of JNK was markedly upregulated in 3T3-L1 adipocytes after 6 hours culture in the presence of 10 μg/ml tunicamycin. Inclusion of the specific JNK inhibitor SP600125 prevented tunicamycin-dependent phosphorylation of c-Jun.

ER stress impairs insulin signalling in 3T3-L1 adipocytes

To determine if ER stress in 3T3-L1 adipocytes affects insulin signalling we assessed expression and activation of IR, IRS1, Akt/PKB and of ERK1/2. After 18 hours both Thap (Fig. 7A and 8A) and Tu (Fig. 8B) strongly reduced the expression and insulin-dependent phosporylation (Fig. 7A) of the IR. Only mild reduction in expression (Fig. 7B and 8B) and insulin-dependent tyrosine phosphorylation (Fig. 7B) of IRS1 was observed while insulin-dependent phosphorylation/activation of Akt/PKB and ERK1/2 was strongly inhibited after 18 hours of

culture in the presence of either Tu or Thap (Fig. 7C). Shorter incubations for 6 hours did not significantly reduce expression of the receptor but clearly reduced the activation of Akt/PKB and ERK1/2 (not shown). In all experiments expression of the ER stress marker Grp78/BiP was determined to control for induction of ER stress.

Constitutive expression of IRS1 or IRS2 and inhibition of JNK counteract ER stress-induced insulin resistance

Next we tested if overexpression of IRS1 or IRS2 under ER stress could rescue insulin-induced activation of Akt/PKB and ERK1/2. 3T3-L1 adipocytes overexpressing IRS1, IRS2 or GFP were incubated in the presence of tunicamycin or thapsigargin for 18 hours and stimulated with insulin for 5 or 20 min. Insulin-induced activation of Akt/PKB and of ERK1/2 under ER stress was strongly reduced in cells overexpressing GFP while cells overexpressing IRS1 or IRS2 showed almost normal activation (Fig. 8A). In contrast, both IRS1 and IRS2 could not significantly improve expression and activation of the IR. Similar results were obtained with CHO-IR cells (not shown). In contrast to overexpression of insulin receptor substrates, inhibition of JNK by SP600125 could only partially counteract Tu- or Thap-induced downregulation of Akt/PKB after five min of insulin stimulation (Fig. 8B). Inclusion of SP600125 did not affect expression of the IR.

ER stress in adipocytes does not inhibit insulin-induced glucose transport/incorporation

Expression and insulin-dependent phosphorylation of AS160 was assessed by Western blotting and glucose transport by measuring the intra-cellular accumulation of 2-Deoxy-D-[1-^{14}C] glucose in 3T3-L1 adipocytes under ER stress or homeostasis. In several series of independent experiments neither Tu nor Thap did significantly impair insulin-dependent glucose transport. Two representative series of experiments are summarized in Fig. 9A. As shown in Fig. 9B for a

single experiment activation of Akt/PKB was strongly reduced. Insulin-induced phosphorylation of AS160 was strongly reduced but not abolished by Tu (Fig. 9C). Total expression levels of AS160 were slightly lower after incubation in the presence of Tu (Fig. 9C). Incubation of 3T3-L1 adipocytes in the presence of the PI3K inhibitor wortmannin resulted in strong inhibition of both, insulin-induced activation of Akt/PKB (lower part of Fig. 4B) and uptake of 2-Deoxy-D-[1-^{14}C] glucose (upper part of Fig. 4B).

ER stress downregulates lipolysis and does not inhibit glucose incorporation in primary adipocytes

Insulin-induced glucose incorporation into white adipocytes isolated from epididymal fat pads of mice was measured. Fig. 10A shows that insulin-dependent glucose incorporation was not changed after 8 hours of incubation in the presence of tunicamycin. Pre incubation for 30 min in the presence of wortmannin resulted in reduced glucose incorporation.

To assess lyposlysis, the concentration of glycerol was determined in culture media of 3T3-L1 and primary adipocytes under homeostasis or ER stress. In a single experiment with 3T3-L1 adipocytes, ER stress was induced using tunicamycin (2 μg/ml) for 18 hours. Isoproterenol (10 μM) was used as a positive control to induce lypolysis. As shown in Fig. 10B Tu and insulin both reduced the accumulation of glycerol in the culture medium to similar levels. Isoproterenol induced the accumulation of glycerol and this increase was inhibited by Tu. Similar results were obtained after the induction of ER stress with Thap (not shown).

Isolated primary adipocytes from mice were incubated in the presence of Tu (10 μg/ml) for four hours to induce ER stress. Like in 3T3-L1 adipocytes, Tu, insulin and their combination significantly reduced (to about 70 %, $p<0.05$) the accumulation of glycerol to comparable levels (Fig. 10C).

ER stress changes the secretion of adipokines from 3T3-L1 adipocytes

To assess if and how ER stress affects the secretion of adipokines from 3T3-L1 adipocytes the accumulation of adiponectin, leptin and IL-6 was measured in supernatants. Fig. 11A summarises the results of seven experiments in which we measured by ELISA adiponectin after incubation in the presence of tunicamycin. ER stress significantly lowered (to 48.9 ± 7 %, $p < 0.05$) the accumulation of adiponectin compared to controls. Leptin and IL-6 were assessed in supernatants of cultured cells by multiplex analysis after short-term (5 hours) or long-term (20 hours) induction of ER stress with either tunicamycin or thapsigargin. Results are shown in Fig. 11B. Tunicamycin and thapsigargin both increased significantly the accumulation of IL-6 after 20 hours (1.91 ± 0.26 fold $p < 0.05$ and 40.7 ± 14.5 fold $p < 0.05$, respectively). After 5 hours, however, no significant increase was found for tunicamycin while thapsigargin did significantly upregulate the secretion of IL-6 (1.47 ± 0.26 fold n.s. and 3.7 ± 1.26 fold $p < 0.05$, respectively). Leptin accumulation was strongly decreased in the presence of tunicamycin after 20 hours (to 30 ± 4 % $p < 0.05$) and thapsigargin after 5 hours (to 76 ± 12 % n.s.) and 20 hours (to 31 ± 16 % $p < 0.05$), respectively, while incubation with tunicamycin for only 5 hours did not significantly change leptin secretion (1.36 ± 0.22 fold n.s.).

Conditioned medium from 3T3-L1 adipocytes under ER stress promotes proliferation of INS-1E β-cells

We assessed proliferation of INS-1E β-cells after conditioning their culture medium with supernatants collected from 3T3-L1 adipocytes under homeostasis or ER stress. 3T3-L1 adipocytes were incubated in the presence of increasing concentrations of Tu (0.2, 2 and 10 μg/ml) for 18 hours to induce ER stress. After thorough washing to prevent carry over of Tu, cells were incubated in Kreb's ringer buffer for 2 hours. INS-1E cells were cultured over night in medium conditioned with these supernatants followed by incorporation of [^{3}H]-thymidine for 4 hours. Conditioned medium derived from adipocytes under ER stress caused a significant (around 15 % in the case of 0.2 and 2 μg/ml of Tu, $p<0.5$) increase in the incorporation of [^{3}H]-thymidine into INS-1E cells compared to control (medium from adipocytes under homeostasis). Four independent experiments were performed and a summary of the results is presented in Fig. 12. Similar results were obtained with Thap (not shown).

Discussion

Repression of insulin signalling by ER stress was described in hepatocytes [2] and it was mainly attributed to activation of JNK leading to serine phosphorylation and downregulation of IRS1. Our findings suggest cell-specific differences in the effects of ER stress. Although ER stress represses insulin signalling also in adipocytes, the underlying molecular mechanisms seem to be different. ER stress only mildly repressed activation and expression levels of IRS1 but in the long term (18 hours) strongly reduced expression of the IR.

Ectopic expression of either IRS1 or IRS2 potently counteracted ER stress-induced repression of insulin signalling in 3T3-L1 adipocytes. This observation was surprising in light of the pronounced reduction of IR and also of IGFR (not shown) expression levels. However, remaining expression might be sufficient to initiate insulin signalling. On the other hand, ectopic expression of receptor substrates might compensate for loss of receptor expression. As shown previously [30,34] both IRS1 and IRS2 can efficiently activate insulin signalling independent of insulin. JNK has been implicated in negative regulation of insulin signalling [4,5] and was shown to cause insulin resistance downstream of the UPR in liver cells [2]. Our results suggest a minor role for JNK in the induction of ER stress-dependent insulin resistance in adipocytes since inhibition of JNK could only partially restore insulin signalling under ER stress.

Despite strongly inhibiting insulin signalling, ER stress did not repress insulin-induced uptake of 2-Deoxy-D-[1-^{14}C] glucose. This surprising finding is at odds with the observed strong impairment of insulin-induced activation of Akt/PKB under ER stress. Glucose uptake to a large extend depends on Akt/PKB [35] and incubation of adipocytes in the presence of the PI3K inhibitor wortmannin resulted indeed in significantly reduced insulin-induced glucose uptake. Our results could thus indicate that ER stress-dependent inhibition of Akt/PKB is not equivalent to inhibition by wortmannin, possibly pointing to specific compartmentalization of Akt/PKB within 3T3-L1 adipocytes. Insulin-induced phosphorylation of AS160 was only partially inhibited under ER stress. AS160 is a substrate for Akt/PKB that links Akt/PKB activation to Glut4 translocation [7-9]. Since it was shown that a number of pathways can regulate the

phosphorylation of AS160 [36], ER stress might promote insulin-induced AS160 phosphorylation via alternative pathways, thereby restoring glucose uptake despite very low levels of activated Akt/PKB. In addition, since non-phosphorylated AS160 acts as repressor of Glut4 translocation, the observed reduction in total expression levels of AS160 under ER stress might contribute to normal insulin-dependent glucose transport. In an earlier study Miller and co-workers [37] had described around 50 % lower expression of *glut4* mRNA in 3T3-L1 adipocytes after incubation with either thapsigarin or tunicamycin. However, such a small reduction in mRNA expression might not lead to significantly less Glut4 protein, which was not determined in the study. Another report even showed increased expression of *glut1* mRNA and protein levels, but no change in *glut4* expression under ER stress in L6 myocytes [38]. Moreover, a recent finding indicates that insulin-stimulated Glut4 translocation is not necessarily proportional to Akt/PKB phosphorylation [39]. We tested insulin-dependent glucose transport and uptake into 3T3-L1 adipocytes and primary adipocytes, respectively, and did not observe any impairment under ER stress. Because glucose deposition as measured in our experiments with primary mouse adipocytes is mostly due to incorporation into lipids [40] insulin-dependent lipid synthesis also appears unaffected under ER stress. This notion is in line with our finding that ER stress downregulated lipolysis and also with recent reports showing that the UPR directly regulates genes responsible for lipid homeostasis in liver [41-43].

ER stress induced remarkable changes in adipokine secretion. Reduction of adiponectin protein secretion under ER stress in our experiments is consistent with published evidence showing reduced adiponectin mRNA accumulation under hypoxia-induced ER stress [44]. Given the generally observed interdependence of cytokine production and secretion it is likely that ER stress leads to global changes in adipokine production and secretion beyond adiponectin, leptin and IL-6. As a consequence obesity-induced ER stress in adipocytes *in vivo* will most likely impact on systemic metabolic regulation. Our experiments with conditioned medium indicate, however, that these changes do not necessarily negatively affect other cell types. We found that proliferation of INS-1E β-cells was increased after conditioning their culture medium with supernatants from ER stressed-adipocytes. Proliferation of β-cells in the face of insulin resistance is key to prevent transition to overt hyperglycemia [45,46]. In this respect, ER stress-induced changes in adipocytes, specifically the observed increase in IL-6 secretion, might help to establish the systemic compensatory state often observed in early, pre-diabetic but insulin

resistant stages. Indeed, a recent report indicates that IL-6 might be required for compensatory β-cell function in mice [47].

In conclusion, we show that adipocytes are severely insulin resistant at the signalling level under ER stress. At the functional level, however, insulin-responsiveness with respect to glucose uptake and lipid synthesis is retained, while the secretion of adipokines is deregulated. As a consequence, obesity-induced ER stress might allow for further increase in fat mass while contributing to the changes in humoral milieu associated with the development of the metabolic syndrome.

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Figure Legends

Figure 6 Induction of ER stress in 3T3-L1 adipocytes. A, Time course experiment showing the induction of ER stress in 3T3-L1 adipocytes by tunicamycin or thapsigargin. Cells were incubated in the presence of 10 μg/ml tunicamycin or 1 μM of thapsigargin for the indicated time, lysed and expression of Grp78/BiP was analysed by Western blotting. B, Tunicamycin induces JNK in 3T3-L1 adipocytes. Cells were incubated for 6 hours in the presence of tunicamycin (10 μg/ml) with or without the specific JNK inhibitor SP600125. Activation of JNK was assessed in lysates by Western blotting using an antibody against the phosphorylated form of c-jun. Expression of actin was assessed as a control.

Figure 7 ER stress represses insulin signalling at different levels in 3T3-L1 adipocytes. ER stress was induced for 18 hours using either tunicamycin or thapsigargin followed by starvation for 2 hours and stimulation with insulin, as indicated. Cells were lysed and insulin signalling was analysed by Western blotting. A, Expression and tyrosine phosphorylation of the IR after 5 or 10 min stimulation with insulin. B, Expression and tyrosine phosphorylation of IRS1 after incubation in the presence of thapsigargin. Cells were stimulated with insulin for 5 min. C, Time course experiment showing insulin-dependent phosphorylation and expression of Akt/PKB and ERK1/2. Insulin was added to a final concentration of 100 nM for 5, 10, 15, 20 or 30 min. In A, B and C expression of Grp78/BiP was assessed as a control for the induction of ER stress (not shown in A and B).

Figure 8 A, Ectopic expression of IRS1 or IRS2 efficiently counteracts ER stress-induced repression of insulin signalling in 3T3-L1 adipocytes. 3T3-L1 adipocytes were transfected with adenoviral constructs encoding IRS1, IRS2 or GFP and cultured for 48 h. Tunicamycin was added to a final concentration of 2 μg/ml and incubation was continued for 18 h. Cells were starved for 2 h prior to the addition of insulin for 5 or 20 min. Expression and activity state of the

IR, Akt/PKB and ERK1/2 were analysed in lysates by Western blotting. Phosphorylation of the IR was visualized using an antibody against phospho-tyrosine. Endogenous and ectopic expression of IRS1 and IRS2 was visualized with specific antibodies or with an antibody against the myc tag. Expression of Grp78/BiP was assessed to monitor the induction of ER stress and actin to confirm that equal amounts of protein were loaded in each lane. B, Inhibition of JNK partially restores insulin signalling under ER stress. ER stress was induced with thapsigargin (300 nM) for 18 hours. The JNK inhibitor SP600125 was used at a final concentration of 30 μM and was added 30 min prior to insulin. After starvation for 2 h and stimulation with insulin (10 nM) for 5 min cells were lysed and insulin signalling was assessed with antibodies against the IR, Akt/PKB phosphor-Akt/PKB. Phosphorylation of c-Jun was assessed to confirm that JNK was indeed repressed in the presence of the SP600125 compound (not shown).

Figure 9 Analysis of glucose transport under ER stress. A, Insulin-induced glucose transport is not affected under ER stress. For the induction of ER stress 3T3-L1 adipocytes were incubated in the presence of 10 μg/ml tunicamycin (n=6) or 600 nM thapsigargin (n=5) for 20 hours. DMSO was used as a control. After starvation for 2 hours uptake of 2-Deoxy-D-[1-^{14}C] glucose was stimulated with 100 nM insulin for 30 min (Tu or Thap were included during starvation and uptake). Protein concentrations in cell lysates were determined and uptake expressed as cpm/μg protein. B, The PI3K inhibitor wortmannin but not ER stress inhibits insulin-induced glucose transport in 3T3-L1 adipocytes. ER stress was induced in adipocytes with tunicamycin (2 μg/ml) for 20 hours. Wortmannin (W) was used at a concentration of 100 nM. Uptake of 2-Deoxy-D-[1-^{14}C] glucose was stimulated with insulin as indicated for 30 min and results are presented as cpm/μg protein. The graph shows a single representative experiment out of three. Induction of ER stress and activation of Akt/PKB in this experiment were assessed by immunoblotting using antibodies against Grp78/BiP and the phosphorylated form of Akt/PKB, respectively. C, ER stress partially inhibits insulin-induced phosphorylation of AS160. ER stress was induced by incubation of 3T3-L1 adipocytes in the presence of 2 μg/ml Tu for 20 hours. Cells were stimulated with 100 nM insulin for 30 min. The occurrence of ER stress and inhibition of insulin signalling were confirmed with antibodies against Grp78/BiP and phospho-Akt/PKB, respectively. Actin was visualized as loading control. Grp78/BiP, phospho-Akt/PKB, AS160 and

actin were detected in total lysates. phospho-AS160 was immunoprecipitated from the same lysates prior to immunodetection.

Figure 10 A, D-[^{14}C] Glucose incorporation into primary adipocytes isolated from mice. Adipocytes from 6 mice were incubated (in triplicates) for 8 hours in Kreb's Ringer buffer containing either DMSO (control) or tunicamycin (Tu, 10 μg/ml). Wortmannin (W, 100 nM) was added to cells incubated in DMSO 30 min prior to the start of incorporation. Incorporation was stimulated with 100 nM insulin for 60 min in the presence of D-[^{14}C] glucose. * $p < 0.05$. B and C, Assessment of lipolysis in 3T3-L1 and primary adipocytes. Glycerol concentrations were measured in supernatants. B, 3T3-L1 adipocytes were cultured in the presence of DMSO (c) or Tu (2 μg/ml) for 18 hours followed by addition of isoproterenol (10 μM) or insulin (100 nM) as indicated for 1 hour. The figure represents one experiment carried out in triplicate. B, epididymal white adipocytes were isolated from four wild type mice and cultured for 4 hours in the presence of DMSO (c) or 10 μg/ml Tu. Insulin (100 nM) was added as indicated for 1 hour. * $p < 0.05$.

Figure 11 Analysis of adipokine secretion under ER stress. A, ER stress represses the production of adiponectin from 3T3-L1 adipocytes. Cells were incubated in the presence of 2 μg/ml tunicamycin or DMSO (as a control) for 20 hours, medium was collected and the concentration of adiponectin was determined by ELISA. Washed cells were lysed and protein content was determined. The graph represents a summary of seven independent experiments. Adiponectin secretion was significantly lower after incubation with tuncamycin (* $p < 0.05$). Induction of ER stress was confirmed by Western blotting with an antibody against Grp78/BiP (not shown). B, ER stress represses leptin but increases the secretion of IL-6 from 3T3-L1 adipocytes. Adipocytes were incubated in the presence of tunicamycin (10 μg/ml) or thapsigargin (600 nM) for 5 hours to induce ER stress. Cells were washed and incubated in Krebs buffer containing BSA and tunicamycin or thapsisgargin for 90 min. Krebs buffer was removed, cells were washed and incubated over night in normal medium containing tunicamycin or thapsigargin. Afterwards medium was collected, cells were washed and lysed for protein determination. Levels of IL-6 and leptin were determined in the Krebs buffer (black bars) and

the medium from the over night incubation (white bars) using multiplex panels. Results are expressed as fold compared to levels determined from controls.

Figure 12 Analysis of proliferation of INS-1E cells. 3T3-L1 adipocytes were incubated in the presence of DMSO (c) or increasing concentrations of Tu as indicated for 18 hours, washed thoroughly, and incubated in Kreb's Ringer for 2 hours. Induction of ER stress was monitored by Western blotting (not shown). INS-1E cells were starved for 4 hours and incubated for 18 hours in conditioned medium. [3H]-thymidine was added for 4 hours. Four independent experiments were performed (* $p<0.05$).

Figures

Figure 6

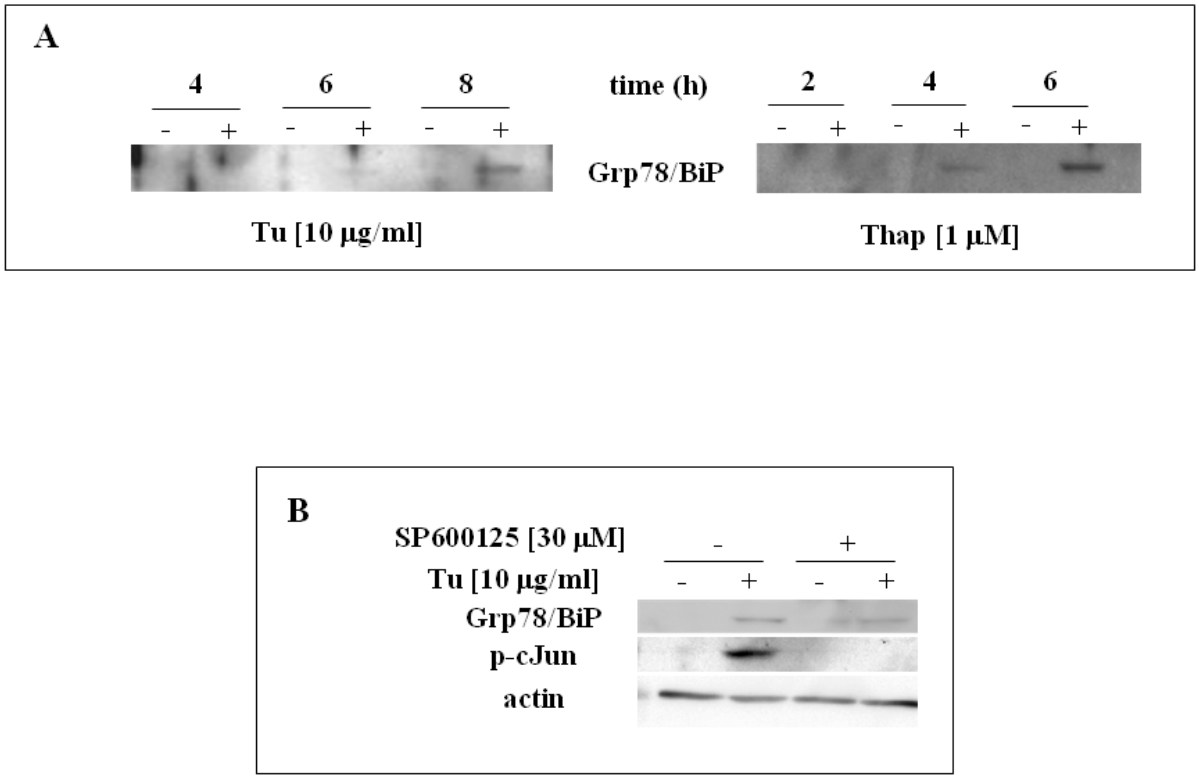
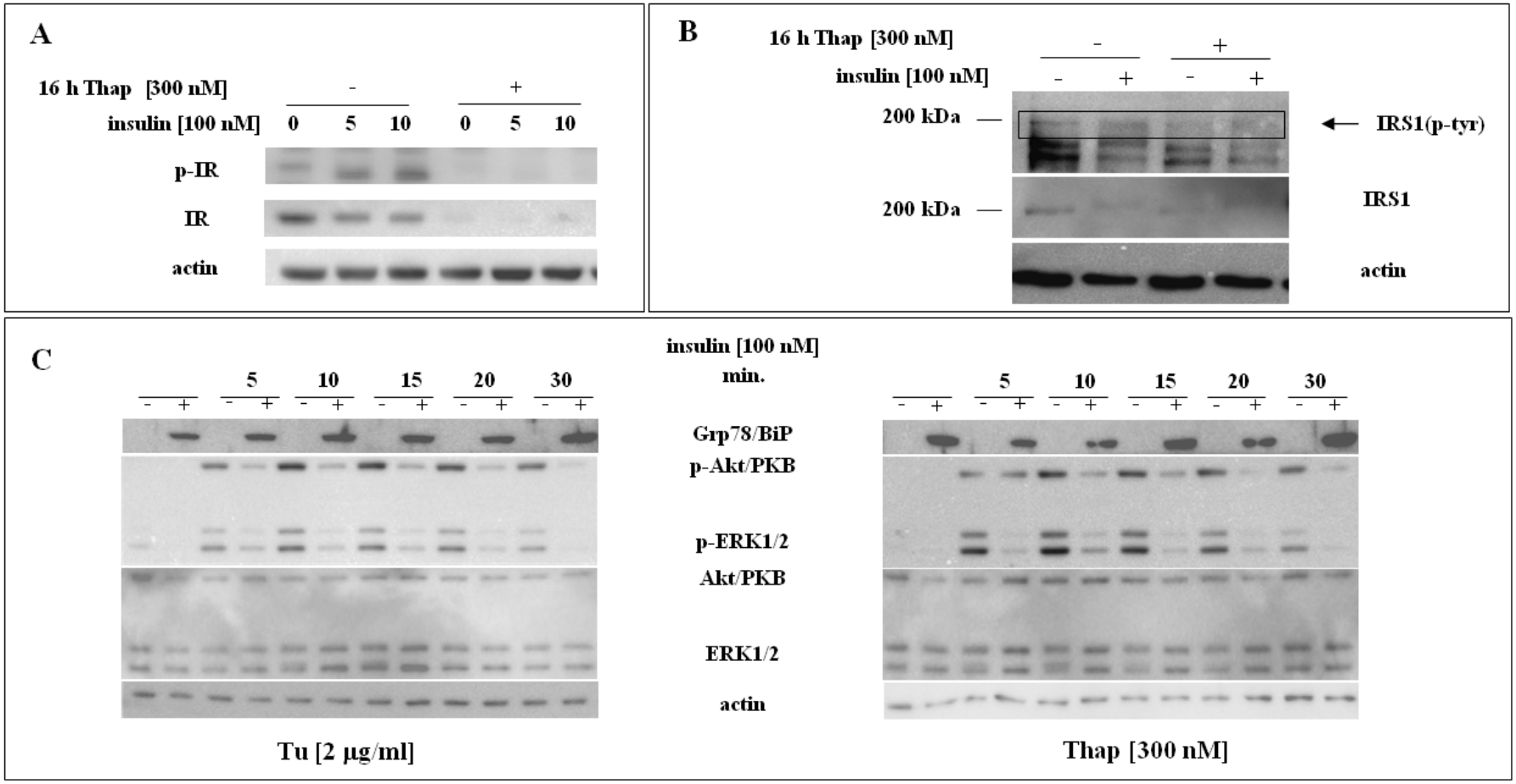


Figure 7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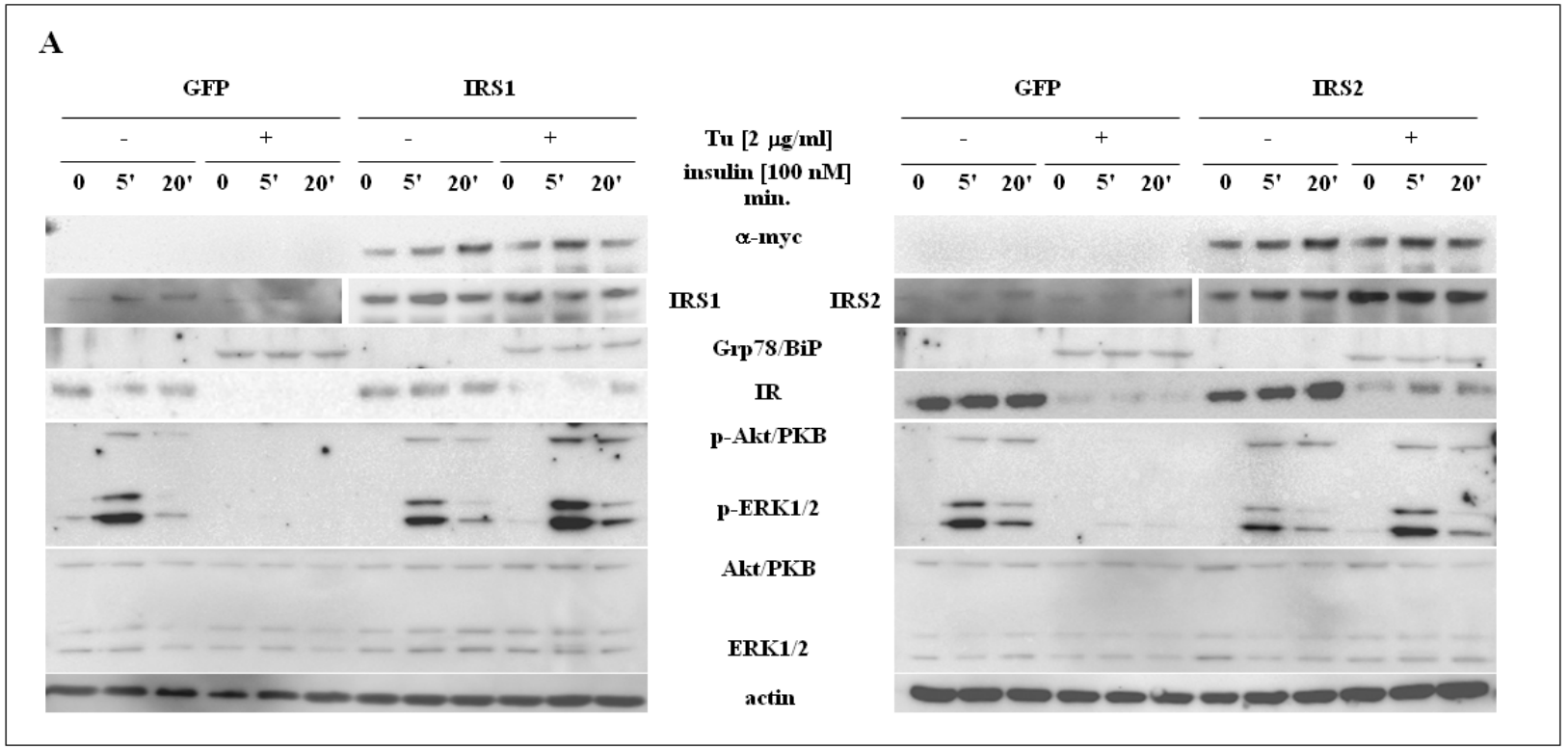
16 h Thap [300 nM]
insulin [100 nM]
0 5 10 0 5 10
p-IR
IR
actin
B
16 h Thap [300 nM]
insulin [100 nM]
200 kDa
IRS1(p-tyr)
200 kDa
IRS1
actin
C
insulin [100 nM]
min.
5 10 15 20 30
Grp78/BiP
p-Akt/PKB
p-ERK1/2
Akt/PKB
ERK1/2
actin
Tu [2 µg/ml]
Thap [300 nM]

Figure 8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GFP
IRS1
GFP
IRS2
Tu [2 µg/ml]
insulin [100 nM]
min.
0 5' 20' 0 5' 20' 0 5' 20' 0 5' 20'
α-myc
IRS1
IRS2
Grp78/BiP
IR
p-Akt/PKB
p-ERK1/2
Akt/PKB
ERK1/2
actin
B
c
Thap
SP600125 [30 µM]
insulin [10 nM]
IR
Grp78/BiP
p-Akt/PKB
Akt/PKB

Figure 9

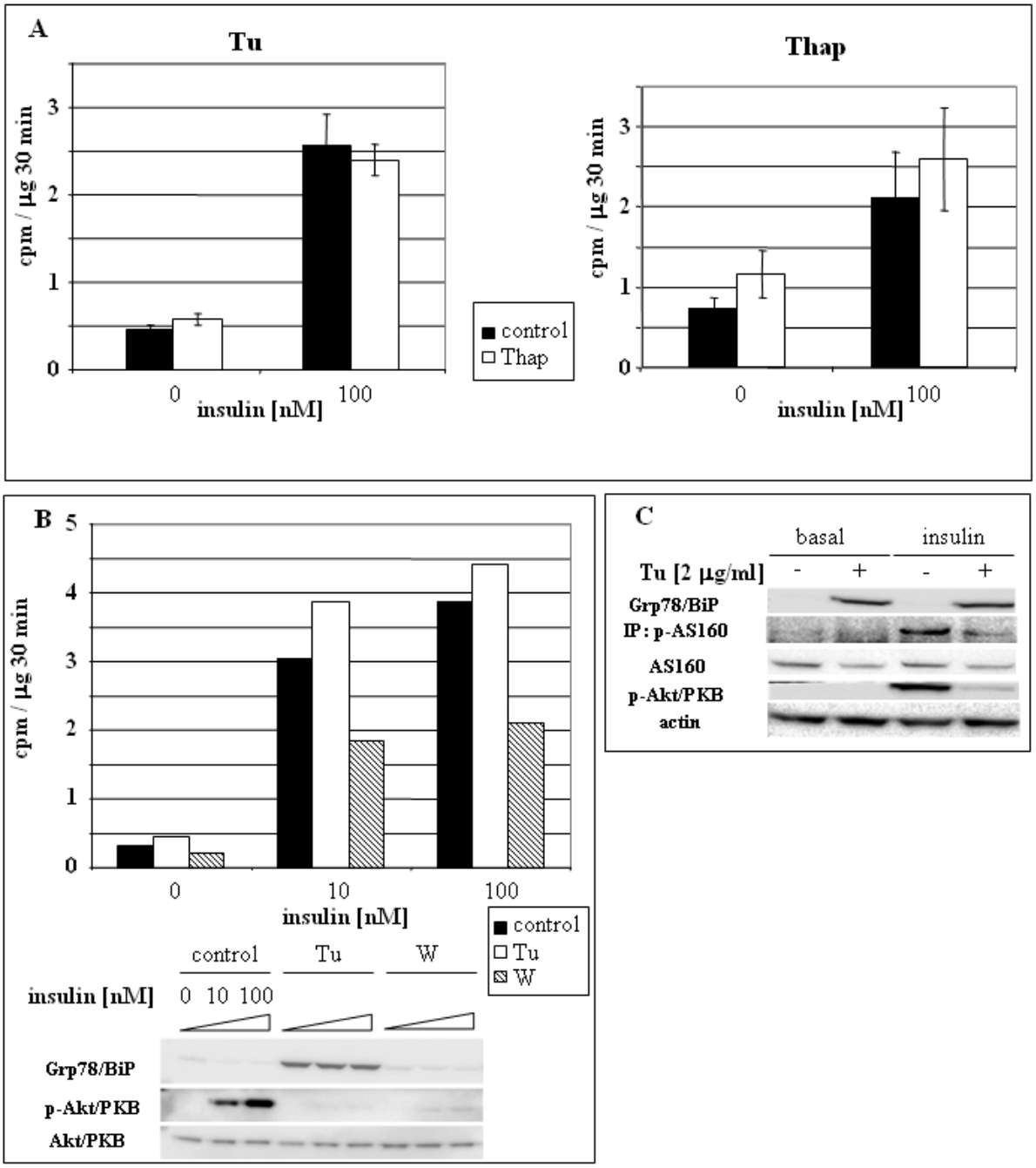

A
Tu
Thap
cpm / µg 30 min
insulin [nM]
control
Thap
B
cpm / µg 30 min
insulin [nM]
control
Tu
W
control
Tu
W
insulin [nM]
0 10 100
Grp78/BiP
p-Akt/PKB
Akt/PKB
C
basal
insulin
Tu [2 µg/ml]
- + - +
Grp78/BiP
IP: p-AS160
AS160
p-Akt/PKB
actin

Figure 10

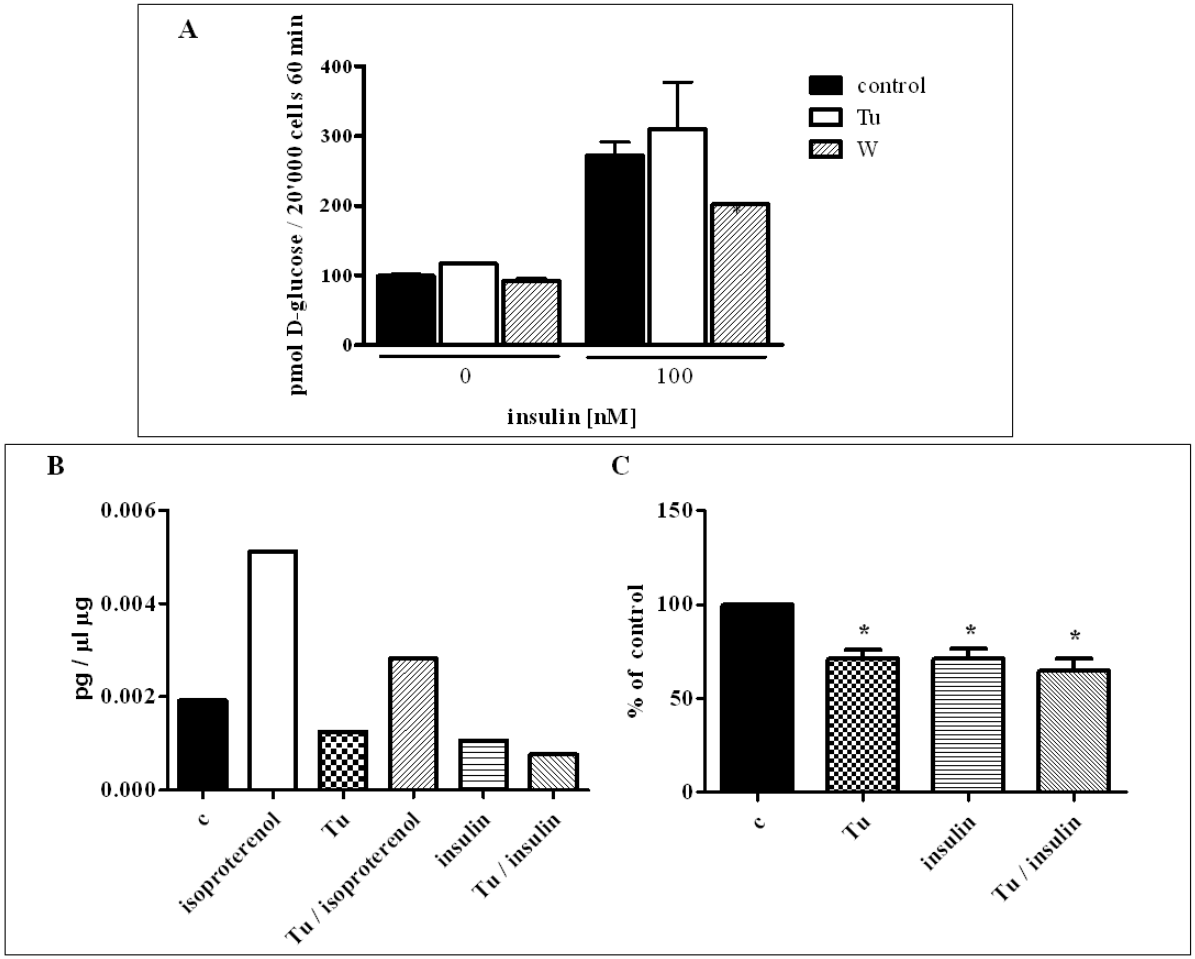

A
pmol D-glucose / 20'000 cells 60 min
400
300
200
100
0
control
Tu
W
0
100
insulin [nM]
B
pg / µl µg
0.006
0.004
0.002
0.000
c
isoproterenol
Tu
Tu / isoproterenol
insulin
Tu / insulin
C
% of control
150
100
50
0
*
*
*
c
Tu
insulin
Tu / insulin

Figure 11

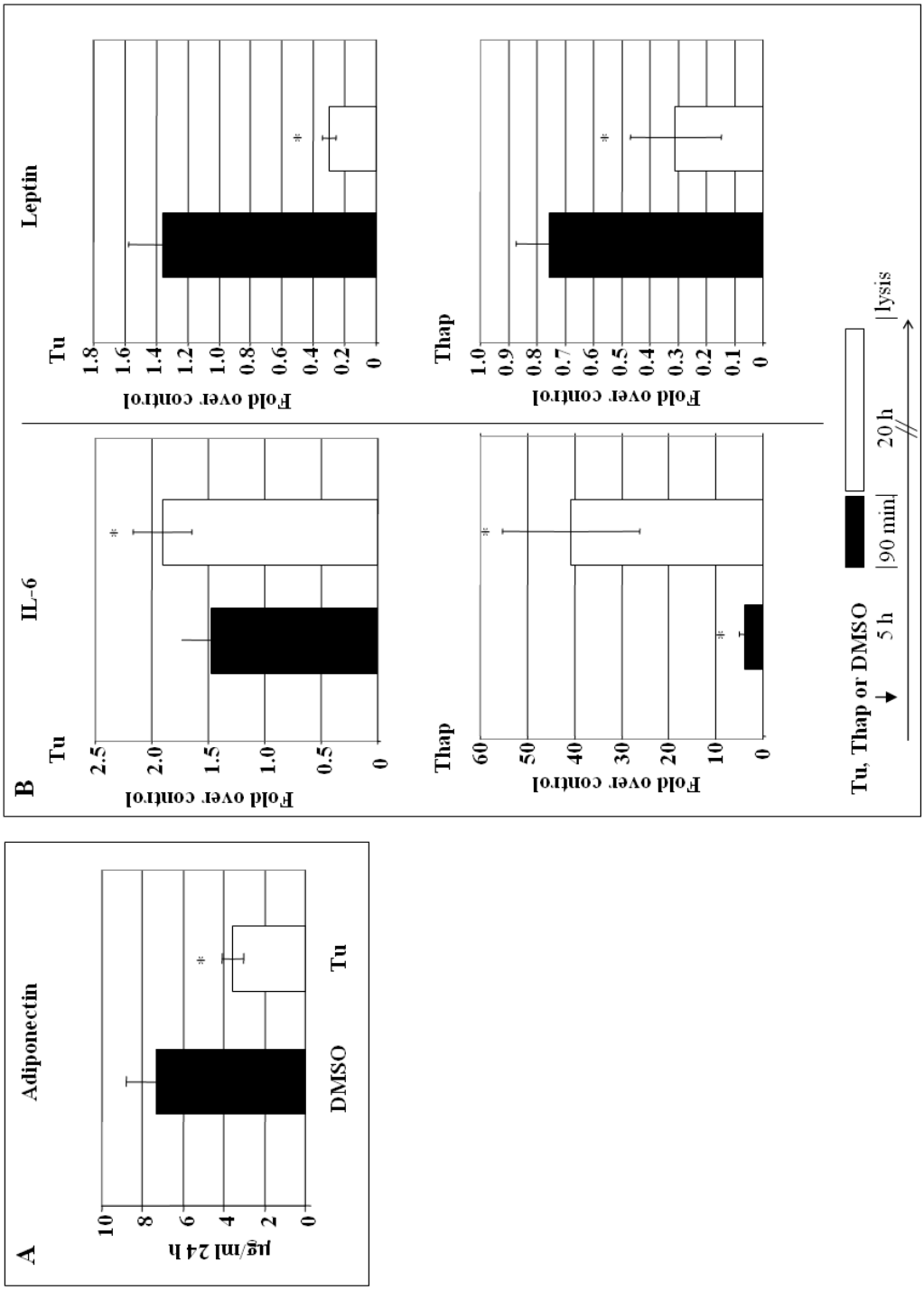
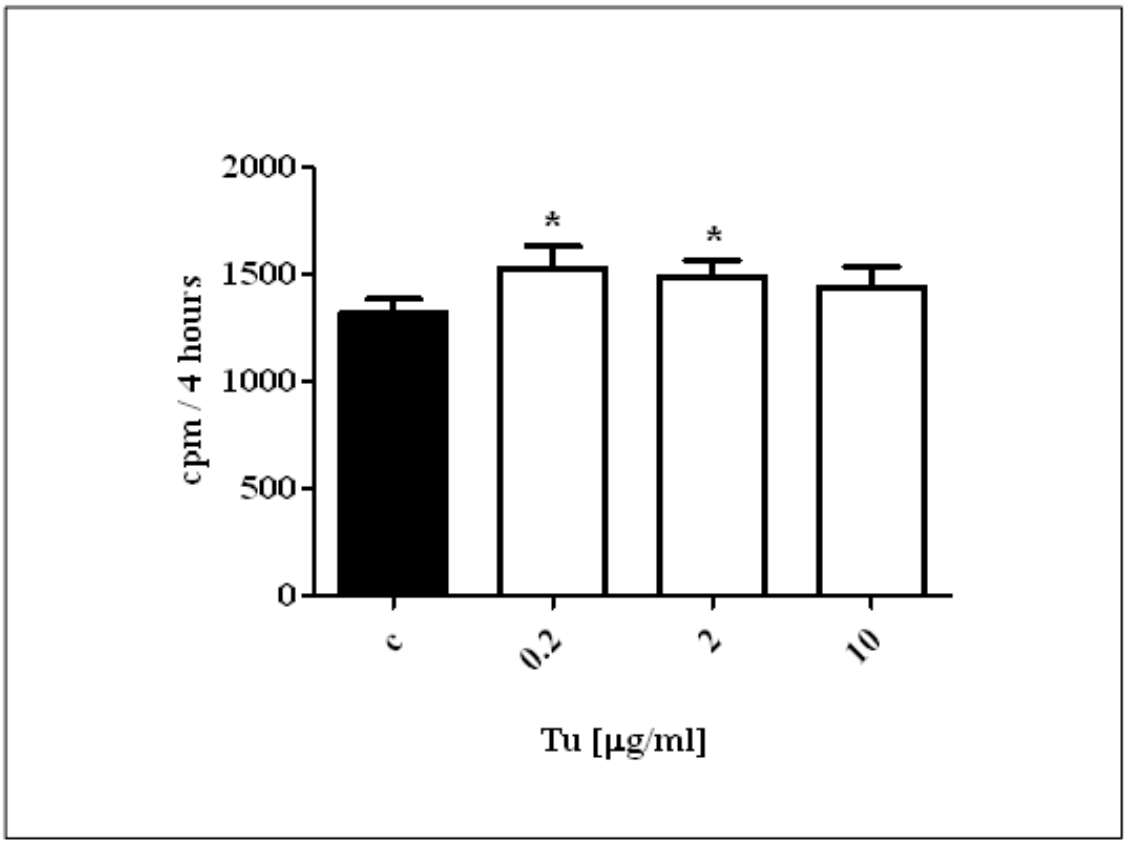

A
Adiponectin
µg/ml 24 h
DMSO
Tu
B
IL-6
Leptin
Tu
Thap
Fold over control
Tu, Thap or DMSO
5 h
90 min
20 h
lysis

Figure 12



2.2 Part 2: The role of Grp78/BiP in insulin signal transduction

2.2.1 Introduction

Grp78/BiP has an ATPase domain in its amino terminus and a peptide-binding domain in its carboxyl terminus that have been modeled on the crystal structures of the heat-shock protein family members HSP70 [1-3]. Grp78/BiP has high affinity to misfolded, underglycosylated or unassembled proteins [4] and it is known to act as a molecular chaperone during the folding and translocation of nascent polypeptide chains [5-7]. The binding of peptides to Grp78/BiP triggers its ATPase activity and ATP binding to Grp78/BiP is required for the dissociation of the Grp78/BiP-peptide complex [8]. As described in the general introduction, Grp78/BiP is well known for its orchestrating role in the activation of the UPR during ER stress [9].

Since it contains a typical cleavable ER-targeting sequence at its N-terminus and an ER-retention sequence (KDEL) at its C-terminus [4], Grp78/BiP is believed to be located in the ER lumen. However, there is growing evidence that some of the ER chaperones are present on the surface of certain cells and can be even released into circulation [10,11] playing a role in cellular physiology. Studies have shown that four hydrophobic domains in Grp78/BiP can form transmembrane helices and that its cytosolic part can offer an interface for cytosolic components which mediate apoptosis [12]. Several studies have shown that newly synthesized Grp78/BiP can escape ER retention and translocate to cell surface by association with murine tumor cell DnaT-like protein 1 (MTJ-1) [13] acting as receptor in mitogensis and proliferation [14,15] or associated with MHC classe I Ages [16] which response for both Dengue virus [17] and coxsackievirus A9 [18]. Furthermore, it was shown recently that thapsigargin-induced expression of Grp78/BiP in PC-3 cells caused its translocation to the cell surface [19].

As mentioned above, a search for new binding partners of IRS proteins had revealed Grp78/BiP [20] and it was determined that the binding between Grp78/BiP and IRS1 occurs between amino acids 336-517 in Grp78/BiP. In IRS1 two regions might contribute to binding [21,22], one is located between aa 270-517 and another one between aa 974-1242 (Figure 13).

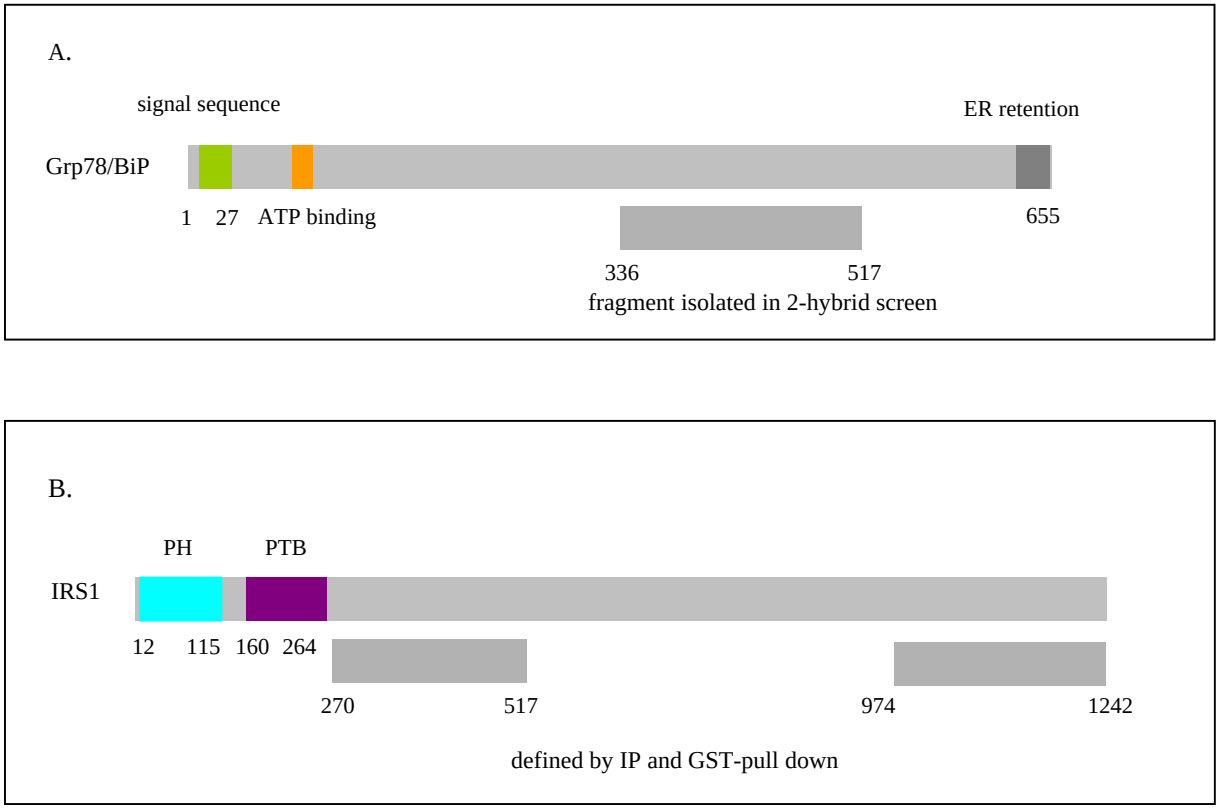


Figure 13. Schematic representation of Grp78/BiP, IRS1 and their respective interacting regions.

A. Grp78/BiP contains a signal sequence (green), an ATP binding site (orange), and an ER-retention sequence (dark gray). Amino acids 336 to 517 bind to IRS. This fragment was identified by yeast 2-hybrid screening. B. IRS1 consists of a PH (blue), a PTB domain (purple) and a C terminal fragment. Fragments spanning aa 270-517 and aa 974-1242 bind to Grp78/BiP and were defined by IP and GST-pull down experiments.

Our discovery that ER chaperone Grp78/BiP can bind to IRS initially raised two questions:

1. Does Grp78/BiP affect insulin signalling?
2. Does binding between Grp78/BiP and IRS regulate insulin sensitivity?

To answer these questions we planned to up- or downregulate Grp78/BiP in target cells and to prevent binding to IRS proteins, respectively. Our strategy included the generation of a number of adenoviral expression constructs. We wanted to induce gain-of-function for Grp78/BiP by overexpression of the full-length wild type protein (Human). Loss-of-function should result after overexpression of mutated versions, e.g. of Grp78/BiP lacking its ATP binding site. Since we were unable to predict in which cellular compartment Grp78/BiP might regulate insulin signalling, constructs were designed to target ectopic expression to various sub-cellular sites, e.g. cytoplasm, ER or even outside of the cell.

While we were performing our first experiments a study was published [23] confirming that ER stress contributes to the development of insulin resistance in liver by activating c-jun N-terminal kinase (JNK), a known inhibitor of IRS proteins. After this finding we immediately adjusted the focus of our study. We first tested the effects of ER stress in our own cellular system of interest (adipocytes, see first part of this thesis) and then tested the role of Grp78/BiP and its binding to IRS. The following second part of this thesis also includes a description of our initial experiments including the expression constructs.

2.2.2 Materials and methods

2.2.2.1 Constructs

A cDNA encoding Human Grp78/BiP was kindly provided by Professor Rick Austin. The sequence of this clone was verified by DNA sequencing. All constructs in Figure 15 are inserted in pCT vector except *Grp78/BiP-9* is inserted in pRRL-CMV-MCS-IRES-GFP vector.

2.2.2.1.1 Grp78/BiP

1**. *Grp78/BiP-1*** (Grp78/BiP wt fused to Myc His Tag).

This clone contains the original Grp78/BiP cDNA in transfer vector containing the cytomegalo virus promoter (*pCMV-transfer*). The stop codon at the 3' end just after KDEL was destroyed by cloning. A Myc His Tag follows KDEL.

```
                                          HindIII
                                  agctggctagttaagcttgccacc

  1 augaagcucucccugguggccgcgaugcugcugcucagcgcggcgcgggccgaggag
      M  K  L  S  L  V  A  A  M  L  L  L  L  S  A  A  R  A  E  E

 61 gaggacaagaaggaggacgugggcacgguggucggcaucgaccuggggaccaccuacucc
      E  D  K  K  E  D  V  G  T  V  V  G  I  D  L  G  T  T  Y  S

121 ugcgucggcguguucaagaacggccgcguggagaucaucgccaacgaucagggcaaccgc
      C  V  G  V  F  K  N  G  R  V  E  I  I  A  N  D  Q  G  N  R

181 aucacgccguccuaugucgccuucacuccugaaggggaacgucugauuggcgaugccgcc
      I  T  P  S  Y  V  A  F  T  P  E  G  E  R  L  I  G  D  A  A

241 aagaaccagcucaccuccaaccccgagaacacggucuuugacgccaagcggcucaucggc
      K  N  Q  L  T  S  N  P  E  N  T  V  F  D  A  K  R  L  I  G
                                  SiGrp78/BiP-1

301 cgcacguggaaugacccgucugugcagcaggacaucaaguucuugccguucaaggugguu
      R  T  W  N  D  P  S  V  Q  Q  D  I  K  F  L  P  F  K  V  V

361 gaaaagaaaacuaaaccauacauucaaguugauauuggaggugggcaaacaaagacauuu
      E  K  K  T  K  P  Y  I  Q  V  D  I  G  G  G  Q  T  K  T  F

421 gcuccugaagaaauuucugccaugguucucacuaaaaugaaagaaaccgcugaggcuuau
      A  P  E  E  I  S  A  M  V  L  T  K  M  K  E  T  A  E  A  Y
```

```
481 uugggaaagaagguuacccaugcaguuguuacuguaccagccuauuuuaaugaugcccaa
     L  G  K  K  V  T  H  A  V  V  T  V  P  A  Y  F  N  D  A  Q

541 cgccaagcaaccaaagacgcuggaacuauugcuggccuaaauguuaugaggaucaucaac
     R  Q  A  T  K  D  A  G  T  I  A  G  L  N  V  M  R  I  I  N

601 gagccuacggcagcugcuauugcuuauggccuggauaagagggaggggagaagaacauc
     E  P  T  A  A  A  I  A  Y  G  L  D  K  R  E  G  E  K  N  I
                ATP binding

661 cugguguuugaccuggguggcggaaccuucgaugugucucuucucaccauugacaauggu
      L  V  F  D  L  G  G  G  T  F  D  V  S  L  L  T  I  D  N  G

721 gucuucgaaguuguggccacuaauggagauacucaucuggguggagaagacuuugaccag
      V  F  E  V  V  A  T  N  G  D  T  H  L  G  G  E  D  F  D  Q

781 cgugucauggaacacuucaucaaacuguacaaaaagaagacgggcaaagaugucaggaaa
     R  V  M  E  H  F  I  K  L  Y  K  K  K  T  G  K  D  V  R  K

841 gacaauagagcugugcagaaacuccggcgcgagguagaaaaggccaaacgggcccugucu
     D  N  R  A  V  Q  K  L  R  R  E  V  E  K  A  K  R  A  L  S

901 ucucagcaucaagcaagaauugaaauugaguccuucuaugaaggagaagacuuuucugag
     S  Q  H  Q  A  R  I  E  I  E  S  F  Y  E  G  E  D  F  S  E

                                                    IRS binding
961 acccugacucgggccaaauuugaagagcucaacauggaucuguuccggucuacuaugaag
    T  L  T  R  A  K  F  E  E  L  N  M  D  L  F  R  S  T  M  K

1021 cccguccagaaaguguuggaagauucugauuugaagaagucugauauugaugaaauuguu
      P  V  Q  K  V  L  E  D  S  D  L  K  K  S  D  I  D  E  I  V

1081 cuuguugguggcucgacucgaauuccaaagauucagcaacugguuaaagaguucuucaau
      L  V  G  G  S  T  R  I  P  K  I  Q  Q  L  V  K  E  F  F  N

1141 ggcaaggaaccaucccguggcauaaacccagaugaagcuguagcguauggugcugcuguc
      G  K  E  P  S  R  G  I  N  P  D  E  A  V  A  Y  G  A  A  V

1201 caggcuggugugcucucuggugaucaagauacaggugaccugguacugcuugauguaugu
      Q  A  G  V  L  S  G  D  Q  D  T  G  D  L  V  L  L  D  V  C

1261 ccccuuacacuugguauugaaacuguggggaggugucaugaccaaacugauuccaaggaac
      P  L  T  L  G  I  E  T  V  G  G  V  M  T  K  L  I  P  R  N
```

1321 acaguggugccuaccaagaagucucagaucuuuucuacagcuucugauaaucaaccaacu
T V V P T K K S Q I F S T A S D N Q P T

1381 guuacaaucaaggucuaugaaggugaaagaccccugacaaaagacaaucaucuucugggu
V T I K V Y E G E R P L T K D N H L L G

1441 acauuugaucugacuggaauuccuccugcuccucguggggucccacagauugaagucacc
T F D L T G I P P A P R G V P Q I E V T

siGrp78/BiP-2
1501 uuugagauagaugugaaugguauucuucgagu*gacagcugaagacaaggguac*agggaac
F E I D V N G I L R V T A E D K G T G N

1561 aaaaauaagaucacaaucaccaaugaccagaaucgccugacaccugaagaaaucgaaagg
K N K I T I T N D Q N R L T P E E I E R

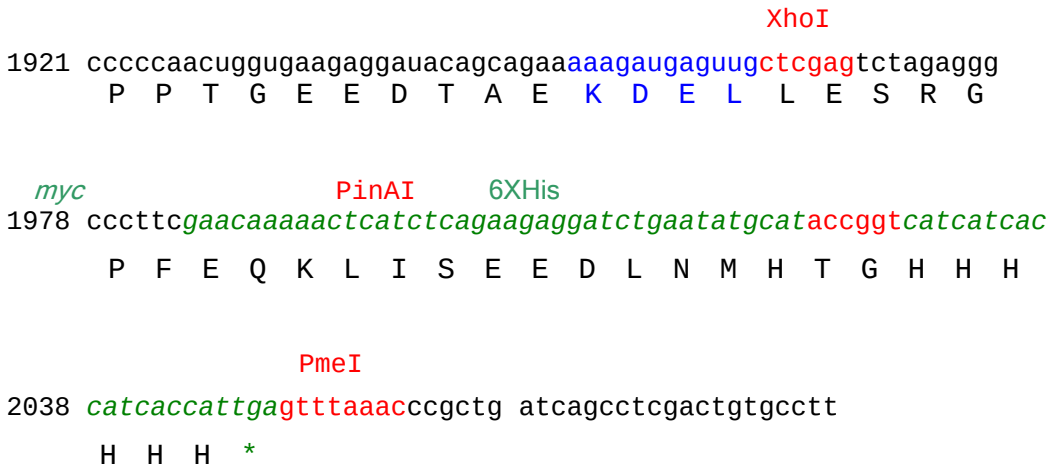
1621 augguuaaugaugcugagaaguuugcugaggaagacaaaaagcucaaggagcgcauugau
M V N D A E K F A E E D K K L K E R I D

1681 acuagaaaugaguuggaaagcuaugccuauucucuaaagaaucagauuggagauaaagaa
T R N E L E S Y A Y S L K N Q I G D K E

1741 aagcugggagguaaacuuuccucugaagauaaggagaccauggaaaaagcuguagaagaa
K L G G K L S S E D K E T M E K A V E E

1801 aagauugaauggcuggaaagccaccaagaugcugacauugaagacuucaaagcuaagaag
K I E W L E S H Q D A D I E D F K A K K

1861 aaggaacuggaagaaauuguucaaccaauuaucagcaaacucuauggaagugcaggcccu
K E L E E I V Q P I I S K L Y G S A G P



2. ***Grp78/BiP-2*** (Grp78/BiP without KDEL containing a Myc His tag)

This construct was generated by Dr. Daniela Dyntar. In *Grp78/BiP-1*, a Myc His tag followed by a stop codon was inserted at 5' of KDEL.

All other constructs as shown in Figure 3 were derived from *Grp78/BiP-1 and Grp78/BiP-2*.

3. ***Grp78/BiP-3*** (Grp78/BiP wt)

Primers (gactggaattcctcctgc and gactcgagctacaactcat) were used to insert by PCR a UAG (STOP) codon 3' to the KDEL sequence in *Grp78/BiP-1*.

4. ***Grp78/BiP-4*** **(**Grp78/BiP with Myc His tag 5' of KDEL)

A Myc His tag was inserted 5' of KDEL. To this end, UAG STOP 5' to KDEL in *Grp78/BiP*-2 was deleted by Quick Change Site Directed Mutagenesis Kit from Stratagene with primers tcatcatcaccatcaccataaagatgagttgtaggttt and aaacctacaactcatctttatggtgatggtgatgatga.

5. ***Grp78/BiP-5, Grp78/BiP-6, Grp78/BiP-7, Grp78/BiP-8*** (mutated ATP binding sites)

ATP binding sites in *Grp78/BiP-1*, *Grp78/BiP-2*, *Grp78/BiP-3* and *Grp78/BiP-4* were mutated (G226D, and G227D) according to [8] to obtain *Grp78/BiP-5*, *Grp78/BiP-6*, *Grp78/BiP-7* and Grp78/BiP-*8*, respectively, by using the Quick Change Site Directed Mutagenesis Kit from Stratagene. The oligos used for this reaction were ctggtgtttgacctggatgacggaaccttcgatgtg and cacatcgaaggttccgtcatccaggtcaaacaccag.

6. ***Grp78/BiP-9*** (Grp78/BiP without N-terminal signal sequence)

The signal sequence of Grp78/BiP extends from aa 1 to aa 18 (http://www.expasy.org/cgi-bin), but the secondary structure from aa 3 till aa 25 is a helix. In order to generate a Grp78/BiP without signal sequence and avoid change the secondary structure, we decided to amplify Grp78/BiP construct from aa 28 to aa 655. Primers gtgatatcaatgggcacggtggtc (contains EcoRV) and gtggatccgcacagtcgab (contains BamHI) were used to amplify *Grp78/BiP-4*, and then cloned the fragment into pRRL-CMV-MCS-IRES-GFP vector.

7. ***Grp78/BiP-10*** corresponds to Grp78/BiP (336-517) and was generated by Bradly Joblin [20].

2.2.2.1.2 IRS

pCT IRS1(270-517) and IRS1(974-1242) were generated previously by Nicola Boschetti (manuscript in preparation).

2.2.2.1.3 *pDsRed2-ER* vector

pDsRed2-ER vector (BD Biosciences) encoded Discosoma sp. Red fluorescent protein (DsRed2) were used for labelling of the endoplasmic reticulum.

2.2.2.2 Adenovirus generation

In brief, constructs were cloned into a transfer vector containing a cytomegalovirus promoter (pCMV-transfer). Recombination with the adenoviral genome in a receiver plasmid (pReceiver) was Cre recombination-based. Following transfection into HEK293 cells the supernatant containing viral particals was collected and purified. For details [24].

2.2.2.3 siRNA

Oligos (corresponding to amino acids 117-124 and 511-518 in wt Grp78/BiP) for hairpin formation [25] were chosen to be complementary to sequences identical in human, mouse, rat and hamster Grp78/BiP. The spacer sequence was 9 nt long. The 5' end contained a BglII restriction site, while the 3' end contained a HindIII restriction site. Synthesized (Microsynth AG) complementary single stranded oligos were annealed and ligated into pSUPER vector (OligoEngine, Inc) containing five thymidines (T5 sequence) as termination signal [26]. Plasmids were finally transfected into mammalian Phoenix-cells and after 3 days the supernatant

contained virus and was used to infect other cells (http://www.stanford.edu/group/nolan/retroviral_systems/phx.html).

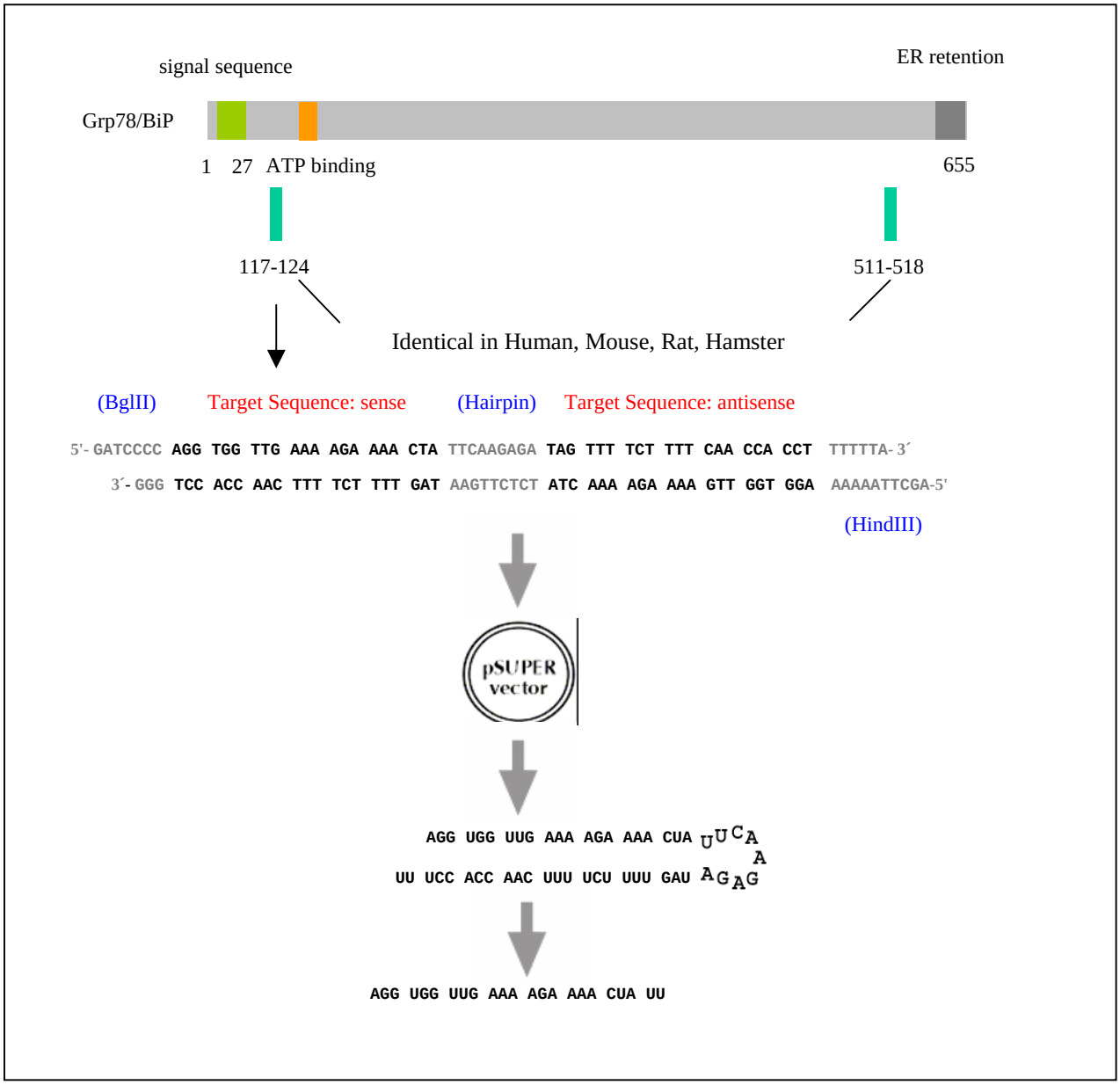


Figure 14. Phoenix retroviral system. Adapted from OligoEngine, Inc.

A target sequence is cloned as a palyndrome into pSUPER vector followed by amplification in DH5α cells. Plasmids are finally transferred into Phoenix cells to produce virus (OligoEngine, Inc).

2.2.2.4 Transfecting plasmids into mammalian cells

OptiMEM (Invitrogen) and Lipofectamin 2000 (Invitrogen) were mixed at a ratio of 15.6:1 and kept for 5 minutes at room temperature. At the same time 2 μg plasmid were mixed with 100 μl OptiMEM. The DNA/OptiMEM and OptiMEM/Lipofectamin 2000 were mixed at a ratio of 1:1 by pipetting up and down 10 times and stored at RT for 15 minutes to allow the formation of micelles. Cells were washed with PBS and 800 μl of pre-warmed OptiMEM was added together with the mixture containing DNA/OptiMEM/Lipofectamin 2000 followed by incubation at 37 °C for 2 hours. Finally, 1 ml of normal culture medium was added.

2.2.2.5 Immunofluorescence staining

Chinese Hamster Ovary cells were plated onto 6 well plates. At 70 % confluence constructs (plasmid DNA) were introduced by lipofectamin-based transfection. After 24 hours culture, cells were washed three times with PBS, fixed in 4 % formaldehyde at 4 °C for 5 minutes and washed three times in PBS. Unspecific binding of antibodies was blocked by incubation of fixed cells in PBS/1 % BSA/0.1 % T-X100 at RT for 1 hour. First and secondary antibodies were incubated for 1 hour. Finally, cells were washed three times with PBS. 100 μl of 100 μg/ml DAPI were added for 10 minutes to stain the nuclei. After washing three times with PBS, the cells were covered in 20 μl antifade Hydromount Aqueous Non-Fluorescing Mounting Medium (National diagnostics, HS-106) under a glass cover slip. Images were acquired and analysed using an Axioplan II system and Axiovision software (Zeiss).

A primary antibody against the Myc tag in combination with an FITC-labelled secondary antibody was used to detect localization of different Grp78/BiP constructs relative to DsRed-ER.

2.2.2.6 Adiponectin

After differentiation, accumulated (24 h) secretion of adiponectin from the 3T3-L1 adipocytes was measured and set as basal. The cells were subsequently transfected with adenoviral constructs to overexpress respective transgenes. After the transfection, the cells were incubated in medium contain 2 μg/ml tunicamycin (TU) or DMSO. The supernatants were collected and the concentrations of adiponectin were measured by using the Mouse Adiponectin ELISA Kit

from AdipoGen (Korea). The expression of the transgene and the induction of ER-stress were confirmed by Western blotting.

2.2.2.7 Antibodies

All antibodies used are described in part one.

2.2.2.8 Methods as described in part 1

Cell culture, differentiation of 3T3-L1 fibroblasts into adipocytes, expression of adenoviral constructs, cell lysis and protein determination, immunoblotting, induction of ER stress, assessment of insulin signalling and 2-Deoxy-D-[1-^{14}C] glucose uptake.

2.2.3 Results

2.2.3.1 Expression constructs of Grp78/BiP

The different Grp78/BiP expression constructs are shown in Figure 15. Since the aim of the study was changed, only three constructs (*Grp78/BiP-3*, *Grp78/BiP-7* and *Grp78/BiP-10*) were used in the later study.

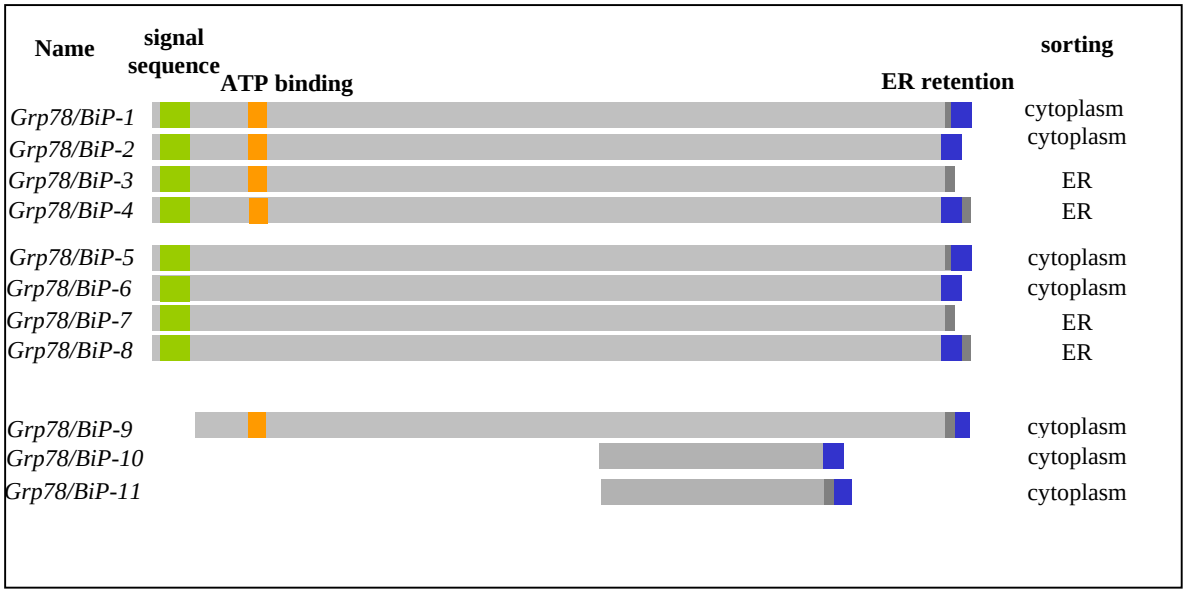


Figure 15. Schematic representation of endogenous Grp78/BiP, Grp78/BiP with mutated ATP-binding site or without KDEL, and the fragment Grp78/BiP(336-517) that binds IRS.

Signal sequence, ER retention signal, ATP binding site and the Myc His tag are indicated in green, dark grey, orange and blue, respectively.

For some of our constructs immunostaining was performed to assess the intra-cellular localization of the fusion proteins expressed. Constructs were co-transfected together with the ER marker pDsRed-ER into CHO-IR cells. Results are shown in Figure 16. As expected, Grp78/BiP-4 was consistently localized to the ER while Grp78/BiP-2 (ΔKDEL) was dispersed within the ER lumen and the cytosol. The result that Grp78/BiP-2 could also be detected in culture supernatant (not shown) indicated secretion. Grp78/BiP-8 (ΔATP) proved to be toxic leading to the destruction of the structure of cells and the Grp78/BiP was no more localized in ER. Grp78/BiP-10 (Grp78/BiP(336-517)) was localized in cytosol.

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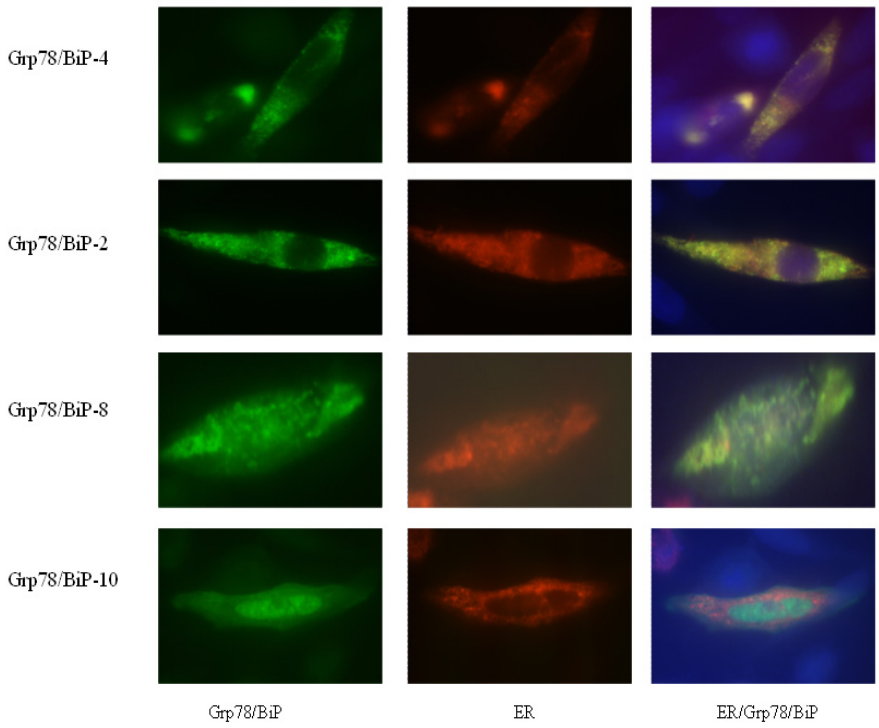

Figure 16. **Immunofluorescence staining to detect fusion proteins in CHO-IR cells.**

Chinese Hamster Ovary cells were co-transfected with pDsRed-ER and expression constructs as indicated. A primary antibody against the Myc-His tag in combination with a FITC-labelled secondary antibody were used to detect localization of different fusion proteins relative to the ER.

2.2.3.2 Grp78/BiP and insulin signal transduction

In order to test if Grp78/BiP plays a role in insulin signalling in 3T3-L1 adipocytes and CHO-IR cells, we overexpressed wt (*Grp78/BiP-3*) or Grp78/BiPΔATP (*Grp78/BiP-7*) under normal conditions or ER stress. In addition to *Grp78/BiP-7,* siRNA was used to induce loss-of-function for Grp78/BiP. We also overexpressed Grp78/BiPΔKDEL (*Grp78/BiP-2*) to test if retention within the ER is required. Insulin signal transduction was analysed by assessing the expression and insulin-dependent activation of IR, MAPK and PKB.

Figure 17A shows the results of one representative experiment (n=3) in which we used lipofectamin to transfect CHO-IR cells. These experiments were performed together with a

master student (Juliana Dias Gonçalves) and only Grp78/BiP wt was used. CHO-IR cells were very sensitive and most of them died due to the combination of lipofectamin transfection and induction of ER stress. For this reason it was only possible to test for the effect of overexpression of Grp78/BiP under homeostasis. Results show that overexpression of Grp78/BiP wt inhibited basal and delayed insulin-dependent activation of IR and MAPK. Activation of PKB was not detectable. The expression of IR and MAPK were not changed.

To determine if overexpression of Grp78/BiP could also influence insulin signalling in 3T3-L1 adipocytes, adenoviral constructs were used followed by induction of ER stress with tunicamycin. In 3T3-L1 adiocytes under normal conditions, overexpression of Grp78/BiP inhibited the expression of the IR and insulin-dependent MAPK phosphorylation but increased PKB activation. Under ER stress, insulin-dependent PKB but not IR and MAPK activation were restored by ectopic expression of Grp78/BiP. Expression of the IR was not detectable in this experiment. These results are presented in Figure 17B. Two independent experiments were performed.

To test if loss-of-function for Grp78/BiP affects insulin signalling, Grp78/BiPΔATP (*Grp78/BiP-7*) was expressed using adenovirus in CHO-IR cells, followed by analysis of insulin signalling under both homeostasis and ER stress. Results are shown in Figure 18. Overexpression of Grp78/BiPΔATP reduced activation of the IR and PKB under homeostasis, however, MAPK activation remained unaffected. Under ER stress the reduction of IR, PKB and MAPK activation were aggravated. Further more, expression levels of Grp78/BiP were very low under both homeostasis and ER stress. This experiment was performed only once.

Two constructs expressing different siRNAs against Grp78/BiP were also transfected into CHO-IR cells. Only siGrp78/BiP-2 slightly downregulated Grp78/BiP under both normal and ER stress (not shown). We could not draw a conclusion with such mild inhibition of the expression of Grp78/BiP.

To test the role of retention of Grp78/BiP within the ER, Grp78/BiPΔKDEL was expressed in CHO-IR cells and insulin signalling was analysed under ER stress or homeostasis. The result is also shown in Figure 18. Compared to GFP control, this construct could repress insulin-dependent IR and PKB activation, but not MAPK activation under homeostasis. Under ER stress, it did not further repress IR, PKB or MAPK activation. In addition, overexpression of Grp78/BiPΔKDEL reduced Grp78/BiP expression.

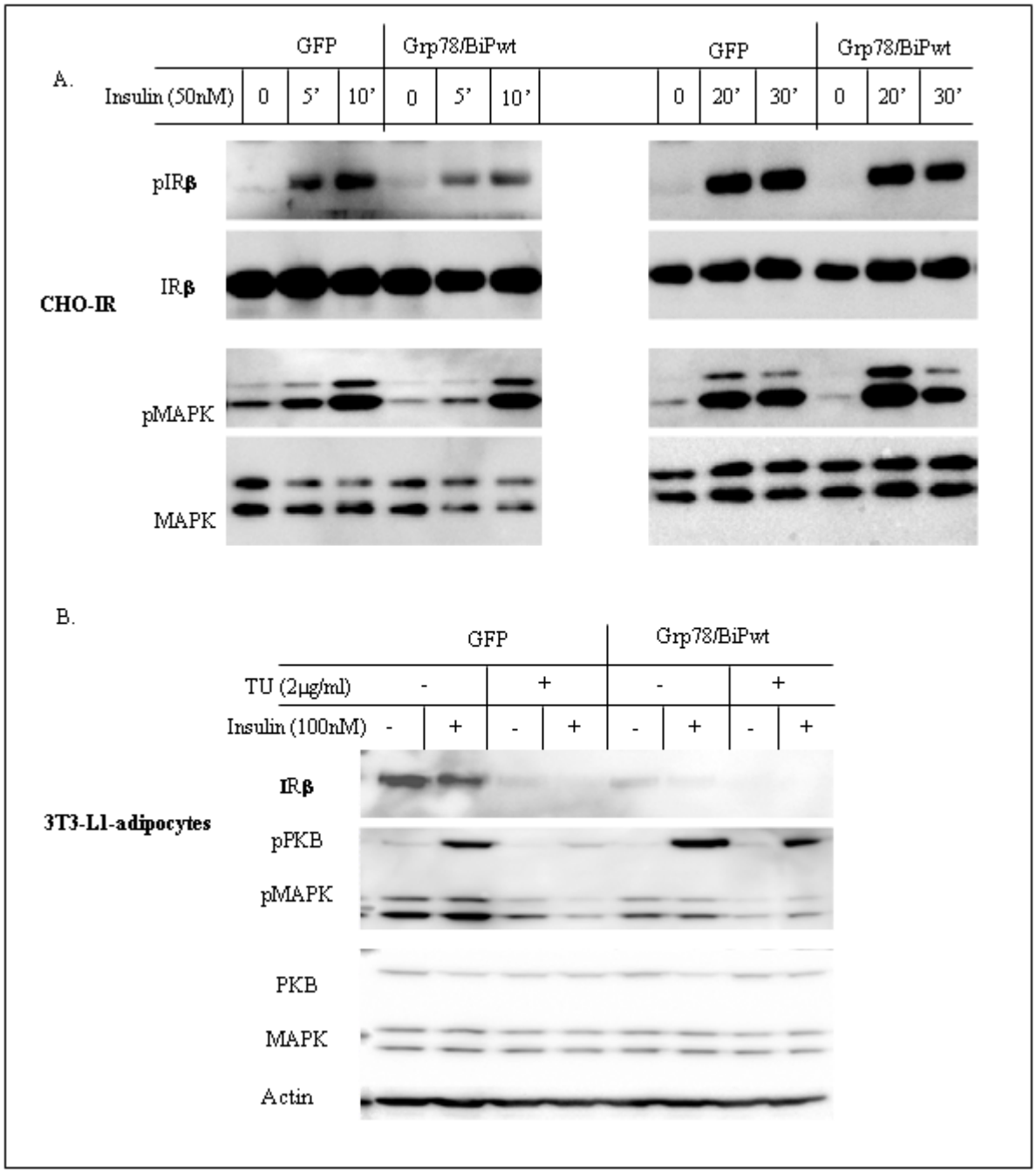

Figure 17. **Western blots showing basal and insulin-dependent signalling in CHO-IR cells (A) and 3T3-L1 adipocytes (B) overexpressing Grp78/BiP**.

A. Chinese Hamster Ovary cells were lipofectamine-transfected for 24 h and insulin was added as indicated. B. 3T3-L1 adipocytes were transfected with adenoviral constructs and cultured for 2-3 days, incubated for 22 hours with either 2 μg/ml tunicamycin (TU) or DMSO followed by 2 hours of starvation. The cells were stimulated at 100 nM of insulin for up to 30'.

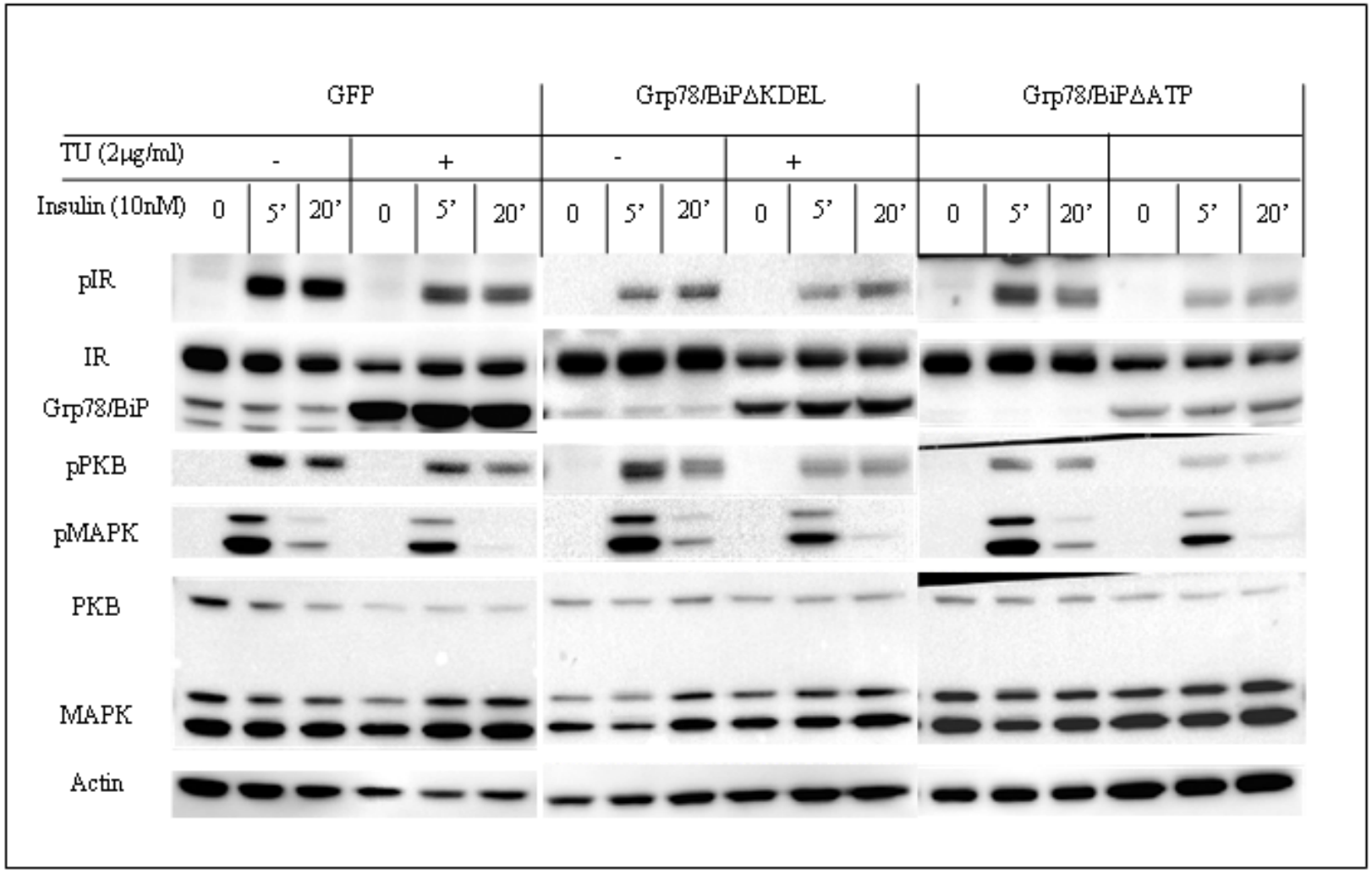

Figure 18. **Insulin-dependent signalling in CHO-IR cells overexpressing Grp78/BiPwt (Grp78/BiP-3), Grp78/BiPΔKDEL(Grp78/BiP-2) and Grp78/BiPΔATP (Grp78/BiP-7) under ER stress or normal condition.**

CHO-IR cells were transfected with indicated adenoviral constructs for 6 hours, followed by incubation in the presence of tunicamycin (2μg/ml) for 20 h. After 1 hour starvation, insulin was added as described. Each blot included a GFP control.

2.2.3.3 Binding between IRS proteins and Grp78/BiP and insulin signal transduction

To inhibit binding between endogenous Grp78/BiP and IRS proteins binding fragments derived from IRS1 were overexpressed in 3T3-L1 adipocytes. We hoped that overexpression of these fragments would compete with endogenous binding. Figure19A shows that expression of either IRS1(270-517) or IRS1(974-1242) alone could not restore PKB activation in 3T3-L1 adipocytes under ER stress. Even more, IRS1(270-517) could repress PKB activation under homeostasis. Co-overexpression of IRS1(270-517) and IRS1(974-1242) was, however, effective and restored PKB activation under ER stress in 3T3-L1 adipocytes (Figure 19B). The expression of fusion proteins was detected using an anti-Myc antibody (not show). Overexpression of Grp78/BiP(336-517) did elevate insulin-dependent PKB activation but further repressed MAPK activation under homeostasis and ER stress in 3T3-L1 adipocytes (Figure 20B). In CHO-IR cells, it could not restore insulin-dependent activation of PKB under ER stress (Figure 20A).

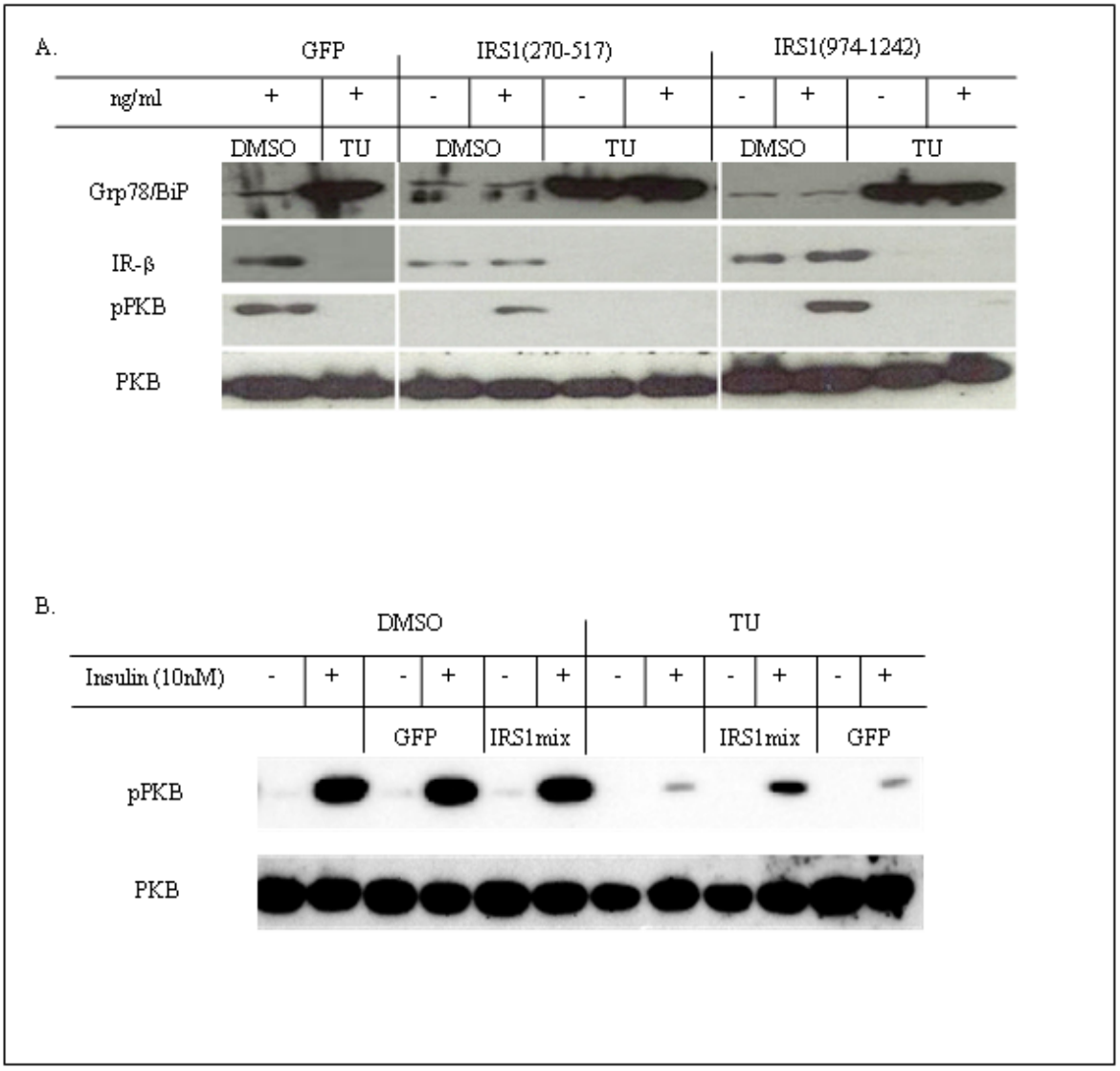

Figure 19. **Western blot showing insulin signal transduction in 3T3-L1 adipocytes expressing the indicated fragments of IRS1.**

Incubation in the presence of TU (2 μg) for 24 hours induced ER stress. Insulin (10 nM) was added to the cells for 30'. A. Overexpression of either IRS1(270-517) or IRS1(974-1242). B. Co-overexpression of IRS1(270-517) and IRS1(974-1242) was labelled as IRS1mix.

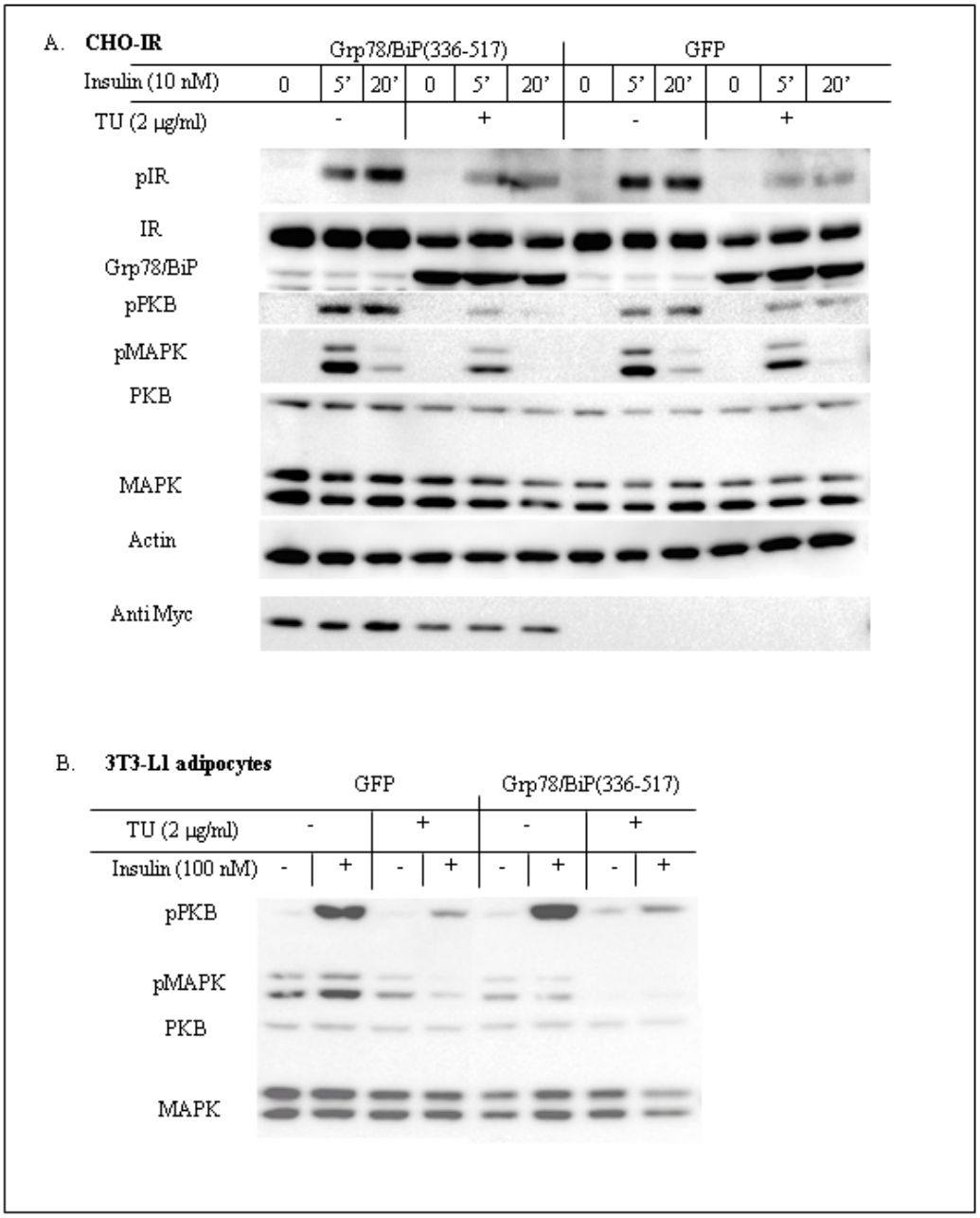

Figure 20. **Western blot showing insulin signal transduction in CHO-IR cells (A) and 3T3-L1 adipocytes (B) overexpressing Grp78/BiP(336-517).**

A. CHO-IR cells were transfected with adenoviral constructs for 16 h followed by incubation in the presence of TU for 20 h. Insulin (10 nM) stimulation was for 5' or 20'. B. 3T3-L1 adipocytes were transfected as indicated for 3 days, incubated in the presence of TU for 24 h and stimulated with insulin (100 nM) for 30'.

2.2.3.4 Does binding between IRS proteins and Grp78/BiP affect adiponectin secretion from 3T3-L1 adipocytes

ER stress significantly lowers the secretion of adiponectin from 3T3-L1 adipocytes (see part 1). To analyse whether binding between IRS and Grp78/BiP regulates the secretion of adiponectin we overexpressed full-length IRS1 or fragments IRS1(270-517) and/or IRS1(974-1242) in 3T3-L1 adipocytes. Figure 21A shows the results of a time course experiment where we measured the accumulation of adiponectin in supernatant by ELISA in the indicated intervals up to 24 hours. In this experiment, adiponectin continuously accumulated over time in a linear manner indicating continuous expression. We hence measured in our experiments the adiponectin that accumulated in supernatant over 24 hours of incubation in the presence of tunicamycin (2 μg/ml) or DMSO. Cells overexpressing IRS1, secreted more adiponectin compared to GFP controls (1.3 fold). Five independent experiments ($p=0.13$) were performed and a summary is shown in Figure 21B. We also expressed IRS1(270-517) or (974-1242) individually or in combination (IRS mix) and measured the adiponectin accumulation. As shown in Figure 21C, IRS1(974-1242) could restored the adiponectin accumulation rate to 1.5 fold over GFP control under ER stress ($p=0.063$, $n=4$).

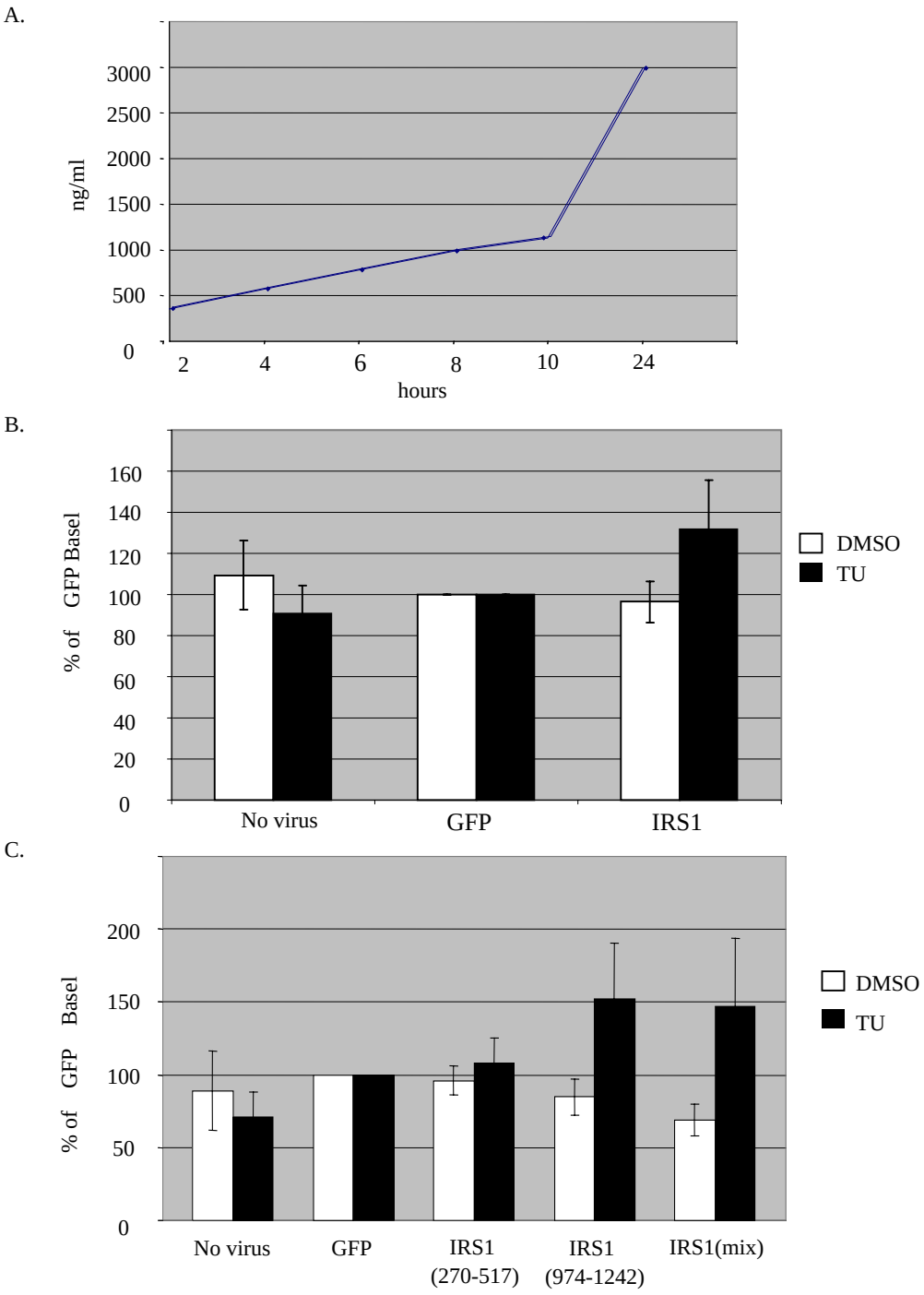

Figure 21. **Adiponectin accumulation in the culture medium of 3T3-L1 adipocytes**.

A. Time course experiment under homeostasis. B. Overexpression of IRS1 in 3T3-L1 adipocytes under homeostasis or ER stress. Results are expressed as mean of five independent experiments. C. Effect of IRS1(270-517) and IRS1(974-1242). Data are presented as mean +/- SE of four independent experiments. In all experiments, secretion of adiponectin from cells expressing GFP was set as basal (100%).

2.2.4 Discussion

Grp78/BiP was discovered in the ER of pre B-lymphocytes bound to and inhibiting the secretion of immunoglobulin heavy chains in the absence of light chains. Most published literature on Grp78/BiP has focused on its function within the lumen of the ER. Grp78/BiP processes nascent secretory and membrane-bound proteins along the protein-folding pathway. Under stress Grp78/BiP binds more permanently to underglycosylated, misfolded or unassembled proteins to retain them in the ER, preventing transport to their functional compartment. Our finding that Grp78/BiP and IRS proteins can bind to each other attracted us to test if and how Grp78/BiP and its binding to IRS might affect insulin signal transduction.

The results of our analysis indicate that there is indeed cross-talk between the ER and insulin signal transduction in adipocytes. The molecular link between insulin signalling and the ER is probably based upon the interaction of Grp78/BiP with insulin receptor substrates.

2.2.4.1 Grp78/BiP in insulin signalling

Grp78/BiP plays similar roles in insulin signalling under homeostasis or ER stress.

As described above, to investigate the role of Grp78/BiP in insulin signalling, we wanted to induce gain-of-function or loss-of-function for Grp78/BiP in both CHO-IR cells and 3T3-L1 adipocytes under homeostasis or ER stress, respectively. To this end we overexpressed full-length wild type Grp78/BiP or Grp78/BiP with a mutated ATP-binding site (Grp78/BiPΔATP), respectively. We also generated expression constructs to downregulate Grp78/BiP by ectopic expression of two different siRNAs.

Throughout this study, we succeeded well in overexpressing wild type Grp78/BiP (Figure 17). However, overexpression of Grp78/BiPΔATP consistently induced changes in cellular morphology and proved to be toxic. Our observation is in line with previous reports that overexpression of Grp78/BiP without ATP-binding domain in monkey cells disrupts the ER [27]. We had not expected such strong and general effects on the ER after ectopic expression of Grp78/BiPΔATP. Furthermore, expression of endogenous Grp78/BiP was only marginally downregulated after expression of our siRNAs. It was not possible to solve these technical difficulties within the time frame of this study and we therefore do not discuss/interpret our results regarding the effects of loss-of-function for Grp78/BiP and insulin signalling.

Under homeostasis, ectopic expression of Grp78/BiP had similar effects in 3T3-L1 adipocytes and CHO-IR cells. Our results indicate that Grp78/BiP can indeed regulate insulin signal transduction. In both cell types, Grp78/BiP reduced insulin-dependent IR and MAPK activation, but enhanced PKB phosphorylation. Due to technical problems, we could not induce gain-of-function for Grp78/BiP in CHO-IR cells under ER stress. But in 3T3-L1 adipocytes, Grp78/BiP restored the activation of PKB, but it further reduced insulin-dependent IR and MAPK activation (Figure 17).

Interestingly, only activation but not total protein levels of the IR was reduced by overexpression of Grp78/BiP. However, we assessed the expression levels of the IR by Western blotting and did not reveal its sub-cellular localization. Like most transmembrane receptors also the IR is produced in the ER. Increased levels of Grp78/BiP under homeostasis might retain the IR in the ER and thereby prevent its translocation to the cell surface where it can be activated by insulin. IR protein levels were reduced under ER stress. In this case, reduction might be due to inhibition of translation downstream of the UPR. It is also conceivable that wrong or missing glycosylation of the IR leads to retention or even the production functionally impaired molecules. Indeed, it has been reported that β chains with mutations in all glycosylation sites sorted properly to cell membrane but had lost the ability to auto-phosphorylate upon stimulation with insulin [28].

The reduction of insulin-induced phosphorylation of the IR in cells overexpressing Grp78/BiP could cause the observed reduction in MAPK activation. On the other hand, previous reports revealed that proteins from the hsp70 family can down regulate MAPK signalling by forming a stable complex [29]. Our results do not allow us to distinguish between these two possibilities.

In contrast to repressing the activation of MAPK, ectopic expression of Grp78/BiP improved activation of PKB under homeostasis and ER stress. This striking difference may results from differential recruitment of receptor substrates to activate PKB or MAPK, respectively. It was shown previously, that insulin receptor substrates can have opposing effects on MAPK and PKB [30]. Furthermore, insulin-dependent activation of PKB has been linked to IRS2 while activation of MAPK was reported to depend on IRS1 in muscle [31]. Clearly, our experiments can not reveal the underlying molecular mechanism but our results suggest that activation of JNK downstream of the UPR might not be the only link between ER stress and insulin signalling.

2.2.4.2 Binding between Grp78/BiP and IRS1 and insulin signal transduction

Following the identification of Grp78/BiP as an IRS-interacting protein we investigated whether the interaction between these two proteins influences insulin signalling. The use of adenovirus-mediated transfection allowed us to overexpress in mammalian cell lines the interaction domains of Grp78/BiP(336-517) and IRS1(270-517, 974-1242). We expected expression of these fragments would compete with endogenous binding between Grp78/BiP and IRS1.

In 3T3-L1 adipocytes, co-overexpression of IRS1(270-517) and IRS1(974-1242) counteracted the repression of PKB activation under ER stress (Figure 19). This finding is in line with the results described in Part 1 of this study where ectopic expression of IRS1 counteracted ER stress-induced insulin resistance. Both results suggest that IRS1 needs to be dissociated from Grp78/BiP for efficient transmission of the insulin signal. Importantly, only co-overexpression of both binding sites could counteract the negative effects of ER stress on insulin signalling. It is therefore conceivable that the two fragments contain non-equivalent binding sites for Grp78/BiP. Given that the two binding fragments are separated by 457 aa, binding of Grp78/BiP might induce significant conformational changes in IRS1 leading to concealment of sites important for IRS function (Figure 21). Alternatively, target sites for JNK might become exposed upon binding of Grp78/BiP.

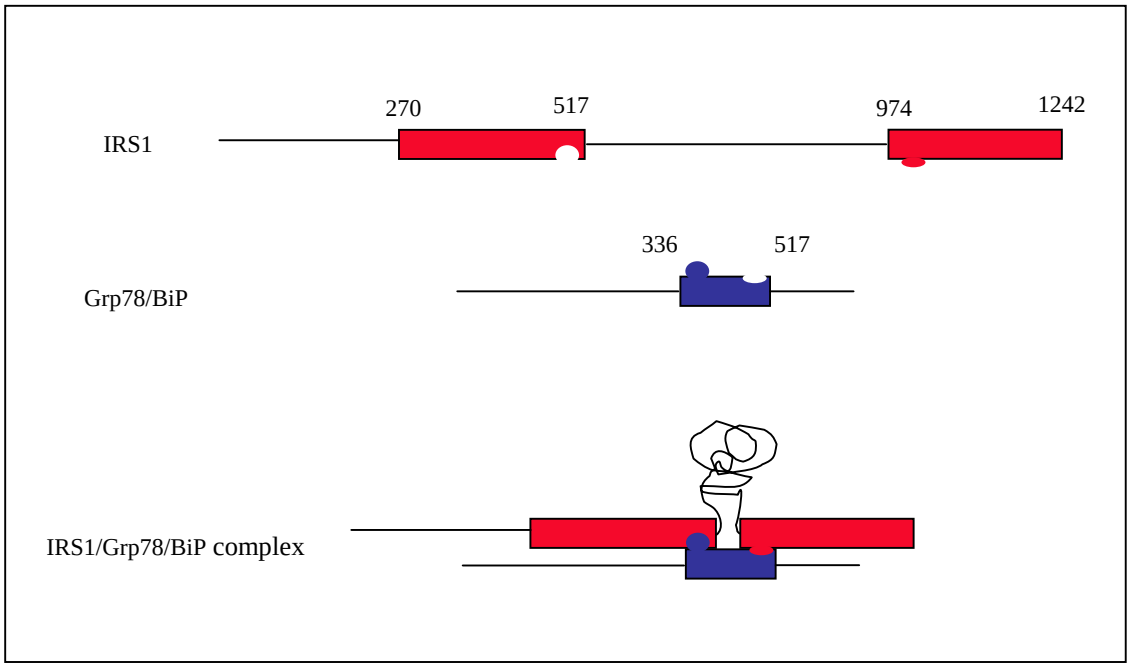

Figure 22. Hypothetical model describing the interaction between IRS1 and Grp78/BiP.
Two binding regions in IRS1 are in red and the Grp78/BiP binding fragment is in blue.

Cells overexpressing Grp78/BiP(336-517) were not rescued from ER stress-dependent repression of PKB and MAPK activation (Figure 20). Overall, overexpression of Grp78/BiP(336-517) showed similar effects as overexpression of full-length wild type Grp78/BiP, under ER stress and homeostasis. This observation suggests that Grp78/BiP(336-517) contains sufficient function of the wild type protein necessary to repress insulin signalling.

2.2.4.3 Compartmentalization of the interaction between Grp78/BiP and IRS proteins

Many studies were focused on the functions of Grp78/BiP within the ER. However, It has been found that in rat pancreatic cells [11], in Meth A sarcoma cells [32], in mature thymocytes [33] and in NG108-15 cells [34], Grp78/BiP is not restricted to the ER lumen, but also expressed on the cell surface. Further more, because Grp78/BiP is associated with plasma membrane and lipid raft domains in liver, it was suggested that Grp78/BiP is a transmembrane protein. The IR and IRS proteins were also reported to be localized in lipid raft microdomains in liver. Another study even showed that IRS1 and IRS2 co-localize with Grp78/BiP in an ER-enriched fraction [35]. In light of all these findings it appears possible that Grp78/BiP can interact with IRS proteins.

Nevertheless, none of our experiments allowed us to determine in which sub-cellular compartment the interaction between IRS and Grp78/BiP occurs.

2.2.4.4 Binding of Grp78/BiP to IRS1 regulates adiponectin secretion from 3T3-L1 adipocytes

Adipokines secreted from adipocytes include adiponectin, leptin, IL-6, TNFα, resistin and many other bioactive proteins. The secretion of these adipokines is changed under obesity [36] and this may contribute to the development of obesity-induced insulin resistance. Adiponectin is exclusively produced and secreted by adipocytes [37]. Animal studies have demonstrated that adiponectin can increase insulin sensitivity, and reduced adiponectin levels are associated with insulin resistance [38]. Adiponectin mRNA levels were found to be lower in type 2 diabetic patients compared to a group of age- and BMI-matched control subjects. In Part 1 of this thesis it was shown that ER stress reduces adiponectin accumulation in 3T3-L1 adipocytes. Overexpression of IRS1, IRS1(974-1242) or IRS1(270-517, 974-1242) could revert the reduction of adiponectin under ER stress (Figure 21). Insulin-stimulated adiponectin secretion from 3T3-L1 adipocytes is controlled by PI3K-dependent signalling [39]. Our finding that co-overexpression of IRS1(270-517, 974-1242) or of IRS1 alone could counteract the reduction of adiponectin under ER stress is in line with our observation that they could also improve PKB activation.

2.2.4.5 Concluding remarks

In conclusion, this study indicates that there is cross-talk between the ER and insulin signalling. This interaction contributes to the regulation of energy balance and affects cellular decisions under both homeostasis and ER stress. The molecular link between insulin signalling and the ER is likely based upon the physical interaction of the ER chaperone Grp78/BiP with insulin receptor substrates. Interestingly, it appears that insulin signalling might be another target of the UPR. But more than that, it is tempting to speculate that the capacity of the ER to produce new polypeptides is co-ordinated with the availability of energy as reflected in the activation of the insulin signalling cascade.

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3 CONCLUSION AND OUTLOOK

Obesity is regarded as the major risk factor for insulin resistance and type 2 diabetes because it is associated with a change in the secretory properties of adipocytes. The endocrine function of fat tissue and its metabolic influence on other tissues has been well documented [1-3].

The finding that obesity can lead to ER stress in liver and adipocytes [4] raised the question whether the ER might link obesity with insulin resistance and the previous publication by Ozcan and colleagues [5] indeed indicated that obesity-induced ER stress could interrupt insulin signalling in liver at the IRS level. More importantly, the occurrence of ER stress was associated with loss of functions required for proper metabolic regulation. Therefore, obesity-induced ER stress was deemed as a major cause of insulin resistance and type 2 diabetes. Despite the proven importance of adipose tissue for systemic metabolic regulation, very little was known on the role of ER stress in adipocytes, prior to our study.

Our specific project originated from the identification of Grp78/BiP as a binding partner of IRS1 and IRS2 (yeast 2-hybrid screeing, [6]). This led us to hypothesise that obesity-induced ER stress could influence insulin signalling and the binding between IRS and Grp78/BiP could be the possible mediator. The objective of this thesis was to investigate the connection between ER and insulin signalling with respect to the regulation of adipocytes function.

ER stress and chronic inflammation

Obesity contributes to the development of type 2 diabetes by promoting chronic inflammation [7] [8-10] possibly by inducing ER stress [11-13]. According to one hypothesis obesity-induced ER stress drives inflammatory signalling cascades by activating IKK, the MAPKs p38 and JNK, and finally transcription factor NF-κB via IRE1 [14,15]. The work presented in this study confirms for the first time that adipocytes under ER stress show increased secretion of a pro-inflammatory factor (IL-6) while an anti-inflammatory factor (Adiponectin) is decreased. Elevated FFA levels in obesity were also considered as a cause of inflammation [16]. However, our results show that lypolysis is reduced by ER stress indicating that ER stress might also counteract the inflammatory process. Inflammatory processes including FFA are well known to inhibit proper β-cell function and regulation. It would be interesting to test if the increase in INS-1E cell proliferation we observed after culture in conditioned medium is due to lower secretion of FFA from adipocytes under ER stress. To this end proliferation of INS-1E might be assessed after conditioned media were supplemented with exogenous FFA.

Adipokines and the regulation of blood glucose homeostasis

A great number of studies have investigated the role of individual adipokines in the development of insulin resistance (reviewed by [1]) and β-cell dysfunction (Reviewed by [17,18]). For example, the study by Elligsgaard et al. indicated that pro-inflammatory IL-6 protects pancreatic α-cells from nutrient-induced apoptosis while it might enhance apoptosis of β-cells [19]. Case studies comparing the obesity-prone Pima Indians to the general Indian population, suggested that people with high adiponectin concentration have less risk to develop type 2 diabetes [20,21]. The plasma concentration of leptin is directly related to the severity of obesity [22]: Increased fat mass correlates with increased leptin levels. Studies have shown that both TNF-α and TNF-α receptor deficient mice are protected from obesity-induced insulin resistance under high fat diet [23] suggesting that TNF-α might constitute the molecular link between obesity and insulin resistance [7]. However, whether the change in secretion of a specific adipokine improves or deteriorates the function of other tissues (e.g. β-cells, liver, or brain) is an intriguing and potentially important question that remains unanswered both *in vitro* and *in vivo*. Considering obesity as a chronic state and taking into account that it not only changes one adipokine at a time, the question is challenging to answer. Indeed and against our expectations, supernatants collected from adipocytes under ER stress stimulated proliferation of INS-1E cells in our thymidine incorporation experiments. The changes in secretion of individual adipokines are difficult to reconcile with this finding. Instead, INS-1E cells appear to integrate over all changes finally showing to improvement of proliferation. According to this view, the often used approach to study the function of individual factors separately might not yield biologically meaningful results. As shown before by others [24] the use of conditioned medium therefore appears as a more promising approach to determine how changes in one cell type could affect another one.

The role of JNK in insulin resistance

JNK is an evolutionarily conserved stress-activated protein kinase which can be induced by environmental insults. It has already been shown that increased JNK activity is associated with type 2 diabetes in humans [25]. In this study, we show that JNK plays a role in ER stress-induced insulin resistance in adipocytes. The previous finding that deficiency for JNK1 in adipose tissue increases hepatic insulin sensitivity under high-fat diet through down-regulation of circulating levels of IL-6 [26] supports the result from this study that ER stress induces JNK activation and elevates IL-6. Interestingly, JNK activation seems to have context-dependent consequence for the cells. It was reported that moderate activation of JNK signalling can result in increased stress tolerance and extended lifespan in flies and worms [27,28]. This indicates that activation of JNK can be protective. However, higher activation of JNK by chronic and serious stress can abolish insulin/insulin-like growth factor signalling (IIS) in flies resulting in developmental delay and metabolic disorder such as hyperglycemia [29]. This indicates that the progression to the pathological state depends on the degree of stress (Figure 23, reviewed by [30]). This study was limited by the use of SP600125 to inhibit JNK. A more specific inhibitor of JNK was not investigated. SP600125 is an ATP-competitive reversible inhibitor and targets all the three different isoforms of JNK. At higher concentrations, it also inhibits other protein kinases upstream of JNK, namely MKK3 and MKK6 (reviewed by [31]). Future experiments to study the role of JNK downstream of ER stress in adipocytes should use the specific peptide inhibitor of JNK, called JNKi [32].

Interaction between Grp78/BiP and IRS links ER and insulin signalling

The results from this study reveal that the interaction between Grp78/BiP and IRS constitutes a molecular link between the ER and insulin signalling. It also validates our approach to search (by yeast 2-hybrid screening) for binding partners of IRS proteins to better define insulin signalling. It is essential to further characterize and more precisely map the binding sites between IRS and Grp78/BiP in later work because knowledge of these residues might pave the way for a new strategy to modulate insulin signalling using competitive peptides, also in the context of insulin resistance. Technically, binding peptides could be mapped in pulldown experiments, tested in suitable bioassay by ectopic expression and finally peptides might be fused to the 10 amino acid

HIV-TAT sequence to confer membrane permeability [33] as a prerequisite for their use *in vivo* to counteract insulin resistance or β-cell failure.

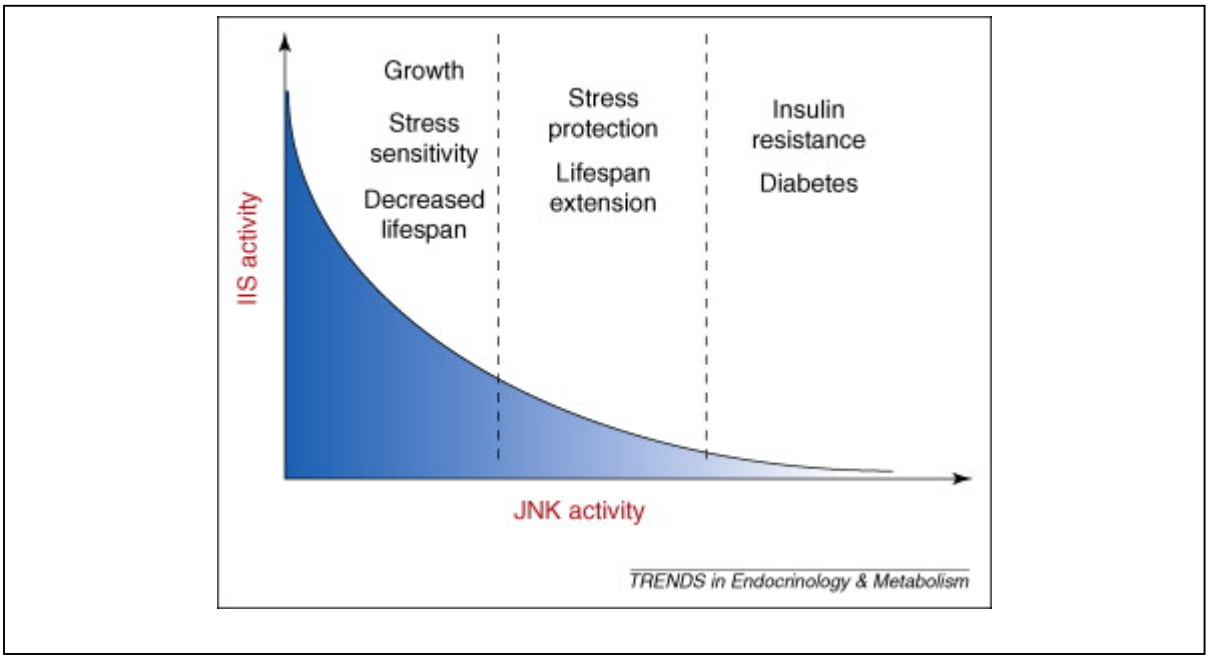

Figure 23. Relationship between JNK and IIS activity, lifespan and metabolic homeostasis.

Moderately increased JNK signalling increases lifespan by reducing IIS activity. In conditions of chronic or excessive JNK activation, however, strong repression of IIS activity results in systemic insulin resistance, promoting diabetes and metabolic syndrome**.** Adapted from [30].

To conclude we could draw a new picture of how ER stress and the UPR might affect adipocytes:

There is most likely cross-talk between the ER and insulin signalling not only under ER stress but also under homeostasis. This interaction contributes to the regulation of energy balance and affects cellular decisions. This work's major contribution to the diabetic field is to show that ER stress inhibits insulin signalling but does not impair metabolic function of adipocytes. It might even support the regulation of glucose homeostasis. Our findings challenge the idea that obesity-induced ER stress and the UPR contribute to development of peripheral fat insulin resistance.

It is hoped that this thesis has provided useful insight into the role of ER stress observed in obesity with respected to the adipocytes, and furthermore, that this knowledge will be considered when decisions are made for clinical intervention targeted at the ER.

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List of Publicaions

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